Community Perspective

Precision Targeting of Cancer Using CRISPR: An Introduction to Precision KLIPP Therapy

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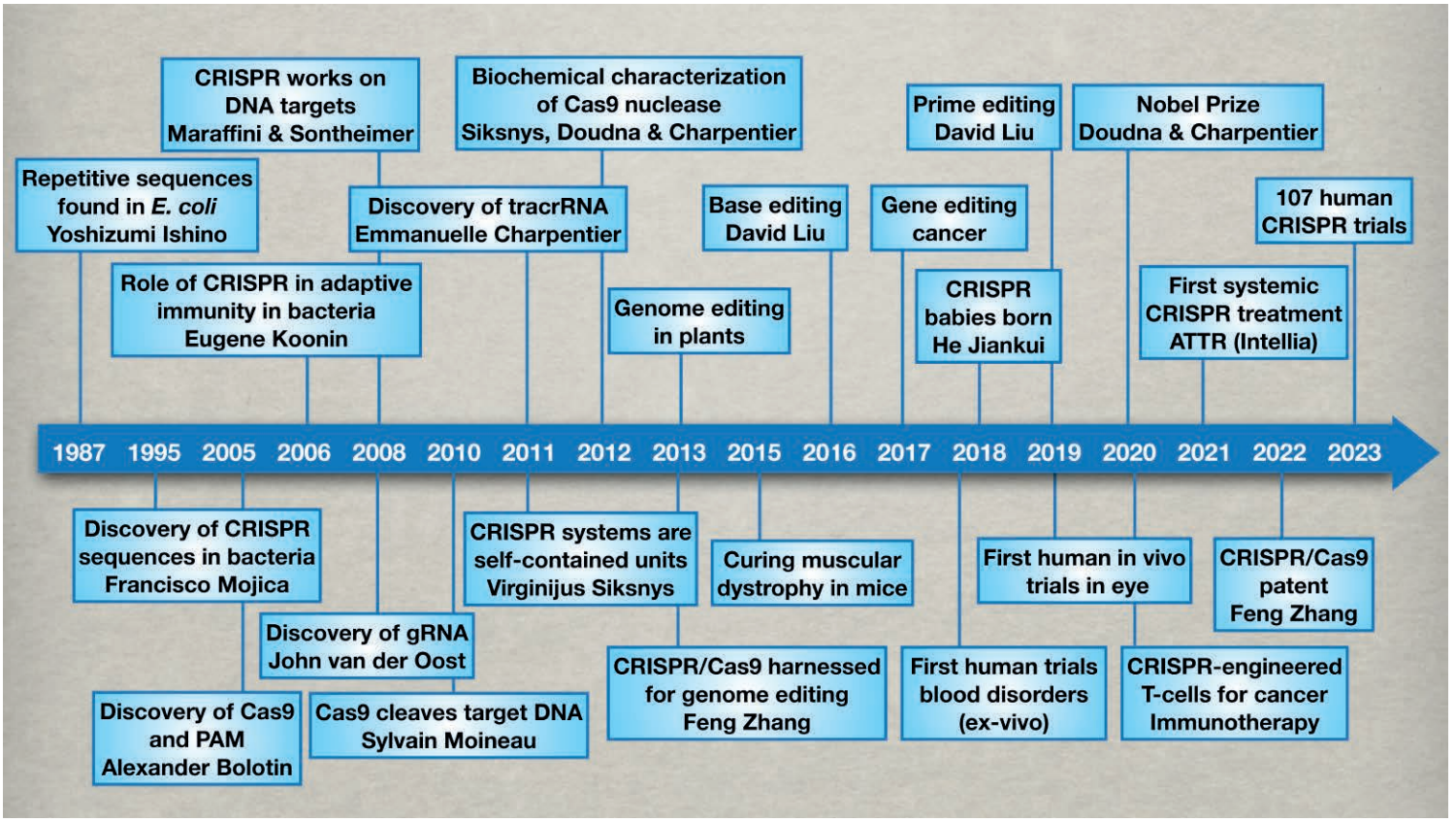


Figure 1: Timeline of the history of CRISPR. Initial discovery of tandem repeat sequences in the bacterial genome in the late 1980s was followed by the identifications of the components of CRISPR. It was further elucidated that CRISPR plays an important role in adaptive immunity in bacteria. The mechanism by which the components of CRISPR works was elucidated by the groups of Charpentier and Doudna in 2012 leading to the Nobel Prize in 2020. The utilization of CRISPR for gene editing was realized and the CRISPR system was re-developed for genome editing without the need for induction of DSBs. The first clinical trial was conducted in 2018 with over 100 clinical trials currently ongoing.

Introduction

According to the author of "The Code Breaker", Walter Isaacson, "The digital revolution was nothing compared to the CRISPR era" (see References 1 and 2, **Inserts 1 and 2**). The economic value of the CRISPR editing market is predicted to surpass $15 billion by 2028 according to Bloomberg, which is quite a remarkable market valuation given that the CRISPR technology is relatively new. This rapid growth of the technology into multiple areas of research, therapeutics, and diagnostics is driven by its programmability, ease of use and of low cost.[1] In contrast to small molecule therapeutic drugs, which typically takes pharmaceutical companies a decade to get to market with a price tag that could hit over a billion dollars, CRISPR therapies can be rapidly manufactured and be used for personalized treatments. For example, CRISPR technologies provide the opportunities to bring sustained cures to patients with rare genetic diseases. Even though these diseases are rare, there are over 7,000 monoallelic diseases that could potentially be cured by CRISPR totaling over 10 million patients in the US alone.[2] Thus, there is a tremendous potential that CRISPR therapies could bring relief and cures for all these patients by leveling the playing field between common and rare diseases. Despite the advantages of CRISPR technologies, however, the cost of personalized medicine may still be prohibitive and may contribute to health disparities due to factors including ability to deliver healthcare and coverage-reimbursement issues.[2-6]

Inset 1: Background on CRISPR as a Molecular Tool

History of CRISPR in Healthcare

The CRISPR field and the many uses for CRISPR in human health is a great example of the paramount importance of federal support for fundamental, curiosity-driven research. Who would have known that studying the innate immune systems of bacteria would one day lead to a health revolution? In 1987, Yoshizumi Ishino and co-workers discovered an unusual genomic structure in *E. coli* consisting of highly homologous sequences of 29 nucleotides arranged as direct repeats with 32 nucleotides as spacing[23] (see timeline in **Figure 1**). Then in 1995, the Spanish researcher Francisco Mojica and co-workers reported on the presence of long stretches of tandem repeats in the genome of Archaea Haloferax and suggested that they are involved in replicon partitioning.[24] It took many years until the components and function of the CRISPR system were elucidated, culminating in the seminal work by Emmanuelle Charpentier and Jennifer Doudna, published in 2012[25] that earned them the Nobel Prize in 2020. With the insight that the CRISPR system could be easily programmed to target any sequence in the genome, a feverish hunt for novel CRISPR systems in other microorganisms and the development of genome editing and new uses for the CRISPR technologies followed. Base editing[26] and Prime editing[27] were developed to capitalize on the programmability of CRISPR but without the need to create DNA double strand breaks which could lead to mutations, chromosome rearrangements and cell death. Several trials are now ongoing both *ex-vivo* and *in vivo* in patients and many more are expected in the coming years.

CRISPR trials and cancer

Many in healthcare anticipate a revolution driven by gene modification tools such as CRISPR, not only in research but also in clinical trials. There were 107 human clinical trials involving CRISPR in various stages of execution listed at the beginning of 2023 (**Table 1**). Of these 107

Table 1: Ongoing CRISPR trials as of January 2023. Cancer and blood disorders are the biggest groupings of patients in these trials

Disease	Phase I	Phase II	Phase III
Cancer	47	20	0
Blood disorders	12	13	14
Other	3	8	0

This data was compiled from https://crisprmedicinenews.com/clinical-trials/, January 2023.

Inset 2: CRISPR/Cas9

The CRISPR system evolved as a bacterial "adaptive immune system" for the defense against viral attacks. When transcribed, these tandem repeats generate RNA that is processed into guide RNAs and used to direct a bacterial DNA endonuclease (Cas9) to complementary DNA sequences of the virus, which is cleaved, and the virus is destroyed. Harnessing the CRISPR system for gene editing, crRNA complementary to the sequence to be edited is generated and when combined with tracrRNA and Cas9 endonuclease, the target DNA sequence is cut into a double strand break (DSB) (**Figure 2**). The cell will attempt to repair the DSB leading to either faithful repair, a mutation (editing) or cell death. Compared to DSBs induced by other sources such as ionizing radiation, DSBs induced by Cas9 are more difficult for cells to repair and result in a higher mutation rate and cell death.[5] The explanation for this difference is most likely that the Cas9 protein has a long resident time at the target site even after the DNA is cut, making it difficult for the DNA repair enzymes to gain access to the damaged site.[6]

trials, 96 trials are for the two disease classes: first, cancer, with 67 trials of which 20 are in phase II evaluation, and second, blood disorders, with 39 trials of which 14 are in phase III testing.

Of other notable trials, one being conducted by Intellia Therapeutics is the first to involve the direct injection of CRISPR reagents into the bloodstream of patients.[7] This trial obtained substantial (>90%) and durable (6 months) knockdown of the target protein transthyretin (TTR) after just a single IV injection of lipid nanoparticles (LNPs) carrying Cas9 mRNA and a *TTR*-targeting sgRNA. These trial results are exciting not only because of the profound reversal of disease progression but also that the treatment was well tolerated, which points to the potential to de-risk the systemic *in vivo* use of LNPs with CRISPR RNAs for this class of human clinical trials.

The 67 cancer trials listed are mainly for blood cancers where CRISPR is used to "*ex vivo*"-edit CAR T-cells, thereby becoming human immune cells genetically engineered to express a chimeric antigen receptor (CAR), an artificial receptor that can identify several types of cancer. By genetically engineering the T-cells to express a CAR, the immune system can find and destroy cancer cells.

To generate CAR T-cells, T-cells are first removed from the patient and either engineered directly with CRISPR[8] or after transfection with tumor-targeting chimeric antigen receptor (CAR).[9] CRISPR technologies are here used to enhance the properties of the T-cells or CAR T-cells by knocking out negative regulators of T-cell persistence and effector function (e.g., PD-1, CTLA-4 and LAG-3). The T-cells or CAR T-cells are then expanded *ex vivo* in the laboratory after which they are transfused back into the patient to fight off the cancer. These personalized cancer treatments have been quite successful, but the price tag could reach half a million dollars for one patient. There are now efforts underway to manufacture "universal" T-cells or CAR T-cells by deriving T-cells from healthy donors and then using CRISPR to edit them to avoid alloreactivity and immunogenicity.[9] Successful implementation of this effort would make this approach available to many more patients and offered at a more reasonable cost.

Precision KLIPP Therapy

Targeting structural variant junctions (SVJs)

Successful cancer therapeutic approaches build on exploiting genetic or biochemical differences between cancer and normal cells. Each approach is based on a means to attack the mechanism by which cancer cells grow or evade or defeat the immune system. The size of the therapeutic window (dose range that provides safe *and* effective therapy) greatly impacts treatment efficacy for such therapy options. Cancer therapies are notorious for inflicting collateral damage to normal tissues.

This serious drawback is especially true for systemic DNA-damaging chemotherapy but even stereotactic radiation therapy, where the radiation-induced double-strand breaks (DSBs) can be concentrated to the tumor, is restrained

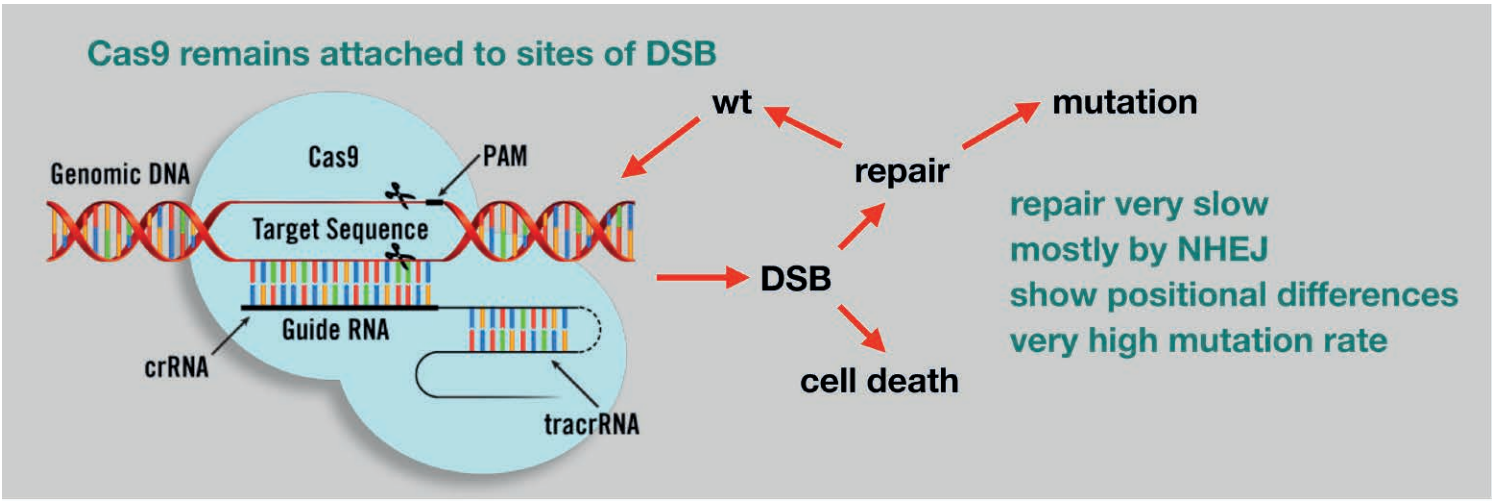


Figure 2: Mechanism of CRISPR/Cas9-mediated DSBs. The endonuclease Cas9 can be targeted to any sequence in the genome by guide RNA and is then activated inducing a DSB. The cell will then attempt to repair the DSB by non-homologous end joining and if successful the integrity of the DNA is restored, or repair may be unsuccessful leading to a mutation or cell death. DSBs induced by Cas9 are difficult to repair because the Cas9 remains attached to the site of the DSB for long periods leading to slow repair, high mutation rates and cell death.

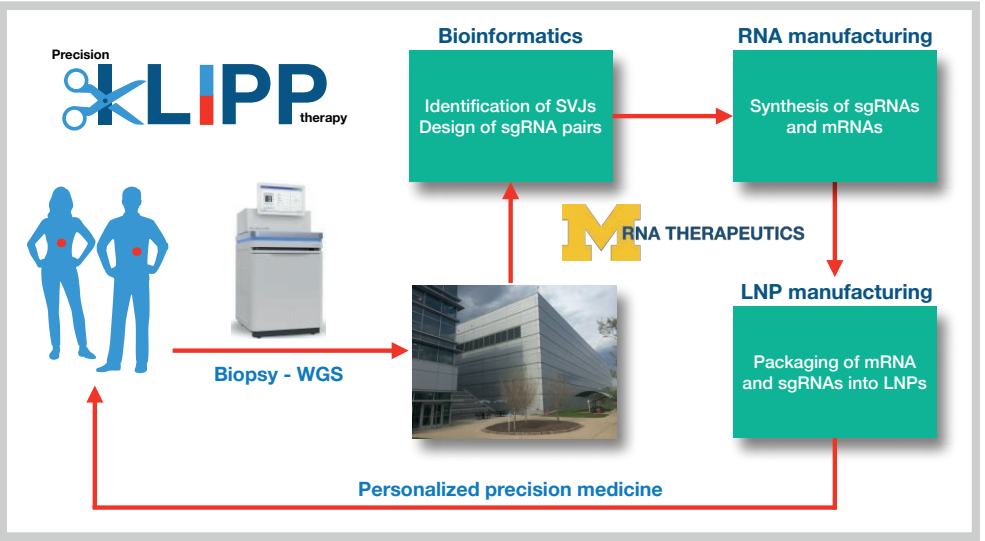


Figure 3: Vision of Precision KLIPP Therapy for cancer treatment. Tumors are biopsied and whole genome sequenced and the sequence data submitted. In the bioinformatic unit, the sequencing data will be analyzed to identify SVJs and to design pairs of SVJ-targeting sgRNAs. The sgRNAs and mRNA are manufactured and then packaged into nanoparticles such as LNPs. The nanoparticles are then sent back to the patient as a personalized precision medicine.

due to dose-limiting toxicity to surrounding normal tissues.

To induce DSBs *precisely* in cancer cells, we have developed a precision CRISPR approach that is based on targeting SVJs that are unique to tumor cells. It has been known for more than 100 years that tumor cells harbor many structural variants that contribute to the early carcinogenic process by activation of oncogenes and suppression of tumor suppressor genes.[10] Recent studies of metastatic cancers found that they harbor a median of about 200 chromosomal structural variants.[11,12] There are two major mechanisms for structural variant formation: replication-based mechanisms, where DNA polymerase jumps to different loci during replication; and fusion-based mechanisms, where a double-strand break is incorrectly repaired, causing distant loci to fuse together.[10]

Developing KLIPP approach

Now, technological advancements in whole genome sequencing (WGS)[13] and CRISPR technologies make it possible for us to target specific structural variants in cancer cells using CRISPR. We have called this approach "KLIPP" which is a Swedish word meaning "cut' and "opportunity." KLIPP uses a split enzyme approach consisting of an inactive Cas9 (dCas9) fused to an endonuclease, Fok1, that needs to dimerize to become active. Pairs of guide RNA (sgRNAs) are generated that bind to both sides of the targeted SVJs, recruiting the Fok1-dCas9 complexes and leading to the activation of Fok1 and the subsequent formation of a DSBs in the cancer cells.[14] We have obtained strong proof-of-concept of KLIPP that we can specifically target SVJs in tumor cells to induce DSBs, detected as γH2AX foci, leading to induction of apoptosis and loss of clonogenic survival. In an orthotopic mouse model of bladder cancer, we were able to eliminate the bladder tumors in over half the mice after targeting as few as two SVJs.

> KLIPP represents a paradigm shift by using two parts of the endonuclease Fok1 fused to dCas9 brought together by pairs of sgRNAs designed to bind to either side of the junctions, thus ensuring that DNA cutting only occurs in cancer cells.

Strategy and advantages of KLIPP

Oncogene amplifications[15,16] and oncogenic fusion genes[17,18] have been targeted directly with CRISPR to reduce oncogene expression, leading to tumor growth inhibition. Since Cas9 endonucleases may also be guided to DNA sequences in normal cells, these approaches are not free from side effects. KLIPP represents a paradigm shift by using two parts of the endonuclease Fok1 fused to dCas9 brought together by pairs of sgRNAs designed to bind to either side of the junctions, thus ensuring that DNA cutting only occurs in cancer cells. In normal cells, these sequences are far apart and therefore the binding of a single Fok1-dCas9 will not lead to the activation of the Fok1 endonuclease.

When brought into clinical practice, we envision that KLIPP reagents can be generated as a precision and personalized medicine for patients in a rapid and cost-effective manner. The first step would involve obtaining tumor and normal DNA from the patient (**Figure 3**). After performing whole-genome DNA sequencing (WGS), SVJs would be identified for that tumor and pairs of specific SVJ-targeting gRNAs would be designed and synthesized.

These sgRNAs would then be combined with Fok1-dCas9 mRNAs, encapsulated into lipid nanoparticles, and delivered to the patient. Therapeutic mRNAs can be delivered by adeno-associated virus (AAV), however, the budding field of lipid nanoparticles (LNP) has emerged to overcome the challenges of specificity and high risk of immune response associated with AAV-delivery. LNPs usually consist of a variety of cationic ionizable lipids to promote endosomal release and encapsulation using cholesterol or polyethylene glycol (PEG)-functionalized lipids to aid in stability and delivery of mRNAs.[19] Since linear mRNAs are highly susceptible to exonuclease-mediated degradation new technologies have been developed to manufacture therapeutic RNA in a circularized form.[20] In addition to enhanced stability, circular RNAs is more compact compared to linear RNA, allowing for more RNA to be packaged inside LNPs.

Plans to introduce KLIPP therapy in the clinic

We intend to initially focus KLIPP therapy on bladder, ovarian and liver cancer, which are cancers that are deadly with few impactful therapeutic approaches available. These cancers are characterized by high levels of structural variants that could be targeted by the KLIPP approach and the modes of delivery of the LNPs are favorable. For bladder cancer, the KLIPP reagents can be delivered directly into the bladder and targeted to the cancer cells using mucosa adhesive formulations of the LNPs.[21] Ovarian cancers often express folate receptors on their cell surfaces and LNPs with PEG-folate will be directed to these cancer cells after IP-injections.[22]

Finally, liver cancers or metastatic cells growing in the liver can be directly targeted with IV-injections of LNPs since LNPs naturally accumulate in the liver.[7] KLIPP has not yet be tested in a clinical trial, but we are gearing up to test KLIPP in a pre-clinical bladder cancer

model in mice. With advances in sequencing technologies and RNA and lipid nanoparticle chemistry, we look forward to showing that KLIPP therapy could have transformative impact on cancer therapeutics in clinical trials and future applications. PMQ

Huibin Yang, MD

Huibin received his MD from Suzhou University, China. He had post-doctoral training at Swiss Federal Institute of Technology in Zurich Switzerland from 1994 to 1998. He joined a pancreatic cancer research team as a research investigator at University of Michigan in 2005. Since 2017 he has been the lead scientist developing the KLIPP therapy in the Ljungman lab.

Radhika Suhas Hulbatte, MS

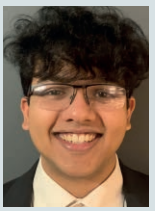
Radhika is Research Lab Specialist in the Ljungman Lab, Radiation Oncology at the University of Michigan. She completed her Bachelor of Engineering in Biotechnology from Visvesvaraya Technological University, India and received a Master of Science in Molecular Biology from Eastern Michigan University. She has worked under the guidance of Dr. Ljungman on the KLIPP team since 2019.

Aadi Shiv Malhotra

Aadi Shiv Malhotra is an undergraduate student at University of Michigan's College of Literature, Sciences, and the Arts (LSA). He is currently pursuing a double major in Biology and Computer Science. His interests include CRISPR research, circular RNA, and computational biology. At Michigan, he is involved in various pursuits such as being a member of the Ljungman lab, MedLaunch, and the Phi Delta Epsilon professional fraternity.

Natalie Gratsch

Natalie graduated with a B.A. in Environmental Science and German from the University of Michigan in 2020. She has been a Research Assistant in the Ljungman Lab since 2020. Natalie is currently a student in the Post-baccalaureate Premedical Program at the University of Michigan Medical School.



Tejas Shivaraman

Tejas is a sophomore at the University of Michigan pursuing a dual degree: B.S. in BCN (Biopsychology, Cognition & Neuroscience) and a B.M.A. in Piano Performance. He has been part of the Ljungman lab since Fall of 2022.

Mario Ashaka, MS

Mario obtained a Bachelor of Science degree in Biological Sciences from Wayne State University in Detroit. From that he gained an interest in the field of genetics which prompted him earn a master's degree from the Department of Human Genetics at the University of Michigan. He accepted a bioinformatic position in the Ljungman lab right after graduating and gaining experience in bioinformatics will positioning him well for the Biotechnology sector which he hopes will be the next adventure in his career.

Mats Ljungman, PhD

Mats is a Professor in Radiation Oncology and Environmental Health Sciences at the University of Michigan. He received his PhD from Stockholm University and performed postdoctoral studies with Dr. Phil Hanawalt at Stanford University. Mats is currently the co-Director of the Center for RNA Biomedicine at University of Michigan. He and his research group developed the nascent RNA Bru-seq technique and the precision KLIPP therapy approach using CRISPR to specifically target cancer cells that they hope to soon bring to the clinic.

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