A Primer on Gene Editing

Myung Soo Kim, Mark Osterman, Shailesh Maingi

Introduction

Gene editing has recently emerged as a revolutionary scientific tool which may be utilized for therapeutic applications such as curing disease, as well as for the creation of animal models for disease research and therapeutics development, agricultural development, genome wide screening, and basic research. In the past two decades, great advances have been made in genome editing technologies through the use of systems, comprised of nucleases combined with target sequence specific DNA binding domains, allowing for direct manipulation of virtually any gene. Prior to the discovery of site specific nucleases, conventional methods only temporarily addressed disease symptoms or randomly integrated therapeutic factors within the genome. Now, scientists are able to more accurately and permanently edit genes by using these nuclease-based systems. This holds great implications for the medical world, as gene editing may be utilized to find cures to diseases through the use of animal models, or even cure genetic disorders. There are four nuclease-based gene-editing systems currently available for use: meganucleases, zinc finger nucleases (ZFNs), transcription activator-like effector-based nucleases (TALENs), and the recently discovered CRISPR/Cas system. Herein, we will discuss the background and history of gene editing, the various gene editing methods including the new CRISPR/Cas9 system, therapies in development, key companies involved in the gene editing space, and challenges faced by the CRISPR/Cas9 system, including ethical and regulatory concerns, focusing primarily on therapeutic and drug development applications of the technology.

Background

Gene therapy is a broad term used to describe any process or strategy for curing or treating disease by modifying, supplying, or blocking genes or gene products. Gene therapy strategies includes the use of viral vectors such as retrovirus, adenovirus, adeno-associated virus (AAV), or the use of non-viral methods such as direct injection, receptor-mediated gene transfer, synthetic oligonucleotides, liposomes, etc. to deliver or modify genes either in vivo or ex vivo[4]. However, these methods suffer from drawbacks such as transient gene expression, transplant-related toxicities, and leukemia induced by the integration of viral vectors into the host genome. Gene editing is a relatively novel tool which may be used for gene therapy. It is much more efficient and specific than traditional tools, allowing for the editing of single nucleotides, and also allows for permanent cures rather than temporary palliative treatments. Gene editing thus represents the future of gene therapy, and, with the advent of the CRISPR/Cas9 technology, has recently come into focus as an exciting area of research with great market potential.

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History of Gene Therapy and Gene Editing

In 1980, Mercol and Cline commenced the first human gene therapy trial to treat β thalassemia patients by transfecting the β globin gene into bone marrow cells, but the protocol was widely viewed as scientifically premature and was stopped on both scientific and ethical grounds. In 1990, Anderson performed one of the first successful gene therapy treatments on a patient with severe combined immune-deficiency (SCID). They successfully added genes coding for adenosine deaminase (ADA), a protein deficient in SCIDs patients, ex vivo into the patient's WBCs and reintroduced the cells into the patients' body which allowed her to survive. There were multiple advances in the 1990s, utilizing hematopoietic stem cells and retroviral vectors for gene therapy. The use of ZFNs was first published in 1991, and they quickly became the preferred method for gene editing for over a decade. However, in 1999, gene therapy suffered a major setback. Jesse Gelsinger, participating in a gene therapy trial using a viral vector to deliver the gene, died due to immunogenicity of the viral vector. This caused the FDA to suspend multiple on-going clinical trials of gene therapy, resulting in significantly impeded research in the area. Subsequently, biopharmaceutical companies have become cautious and suspended their R&D efforts. The FDA eventually eased the ban after regulatory review of the protocol. Research eventually resumed, primarily at academic centers such as Baylor Medical Center, MD Anderson, and Johns Hopkins. These research centers made significant advances in gene editing technologies, specifically in brain delivery, CAR-T cells, deafness, immunogenicity, melanoma, HIV, vision, nanoparticle-based delivery, and more[4]. In 2009, TALENs were discovered, and their benefits over ZFNs and their overall potential resulted in TALENs being named "Method of the Year 2011" by the journal Nature Methods. Between 2013 and April 2014, US companies invested over $600M in the field[5]. Recently, the CRISPR/Cas9 system has been discovered and has brought forth new excitement in the market for gene editing; it was named as "Breakthrough of the Year" in 2015 by Science Magazine, and the MIT Technology Review named it one of their "Top 10 Breakthrough Technologies" in 2014 and 2016. Its simplicity compared to ZFNs or TALENs make it an attractive and exciting new field of research and development. Herein, we will give an explanation of the mechanism of gene editing via targeted nucleases, followed by a brief overview of the different types of targeted nuclease systems used for gene editing: meganucleases, ZFNs, and TALENs, followed by a more thorough evaluation of the CRISPR/Cas9 system.

Mechanisms of Gene Editing via Targeted Nucleases

Targeted gene inactivation, replacement, or addition had been accomplished by homologous recombination (HR); however, its low efficiency in mammalian cells and model organisms posed significant limitations to this technology. The discovery that double-strand breaks (DSBs) increased the frequency of HR by orders of magnitude brought forth targeted nucleases as the preferred method for genetic alteration[6]. Gene editing is currently performed using engineered nucleases (such as meganucleases, ZFNs, TALENs, or CRISPR/Cas9) which create DSBs at specific and targeted locations within the genome. Figure 1 shows how this is done for each nuclease. The DSBs are repaired through either non-homologous end-joining (NHEJ) or homology directed repair (HDR), resulting in specific desired mutations. NHEJ repairs the DSB by directly joining the two DSB ends without requiring a guide template. NHEJ-mediated DSB repair may result in the formation of insertions or deletions, which cause frameshift mutations resulting in nonfunctional proteins; thus, NHEJ may be utilized to suppress gene function. HDR allows for utilization of exogenous DNA templates with sequence homology to the break site to synthesize DNA with the incorporated changes found in the template DNA. This allows for the replacement of genes which may allow for restoration of gene function while preserving physiological regulation of gene expression[7].

There are currently four main types of engineered nucleases used for gene editing: meganucleases, zinc finger nucleases (ZFNs), transcription activator-like effector-based nucleases (TALENs), and the recently discovered CRISPR/Cas system.

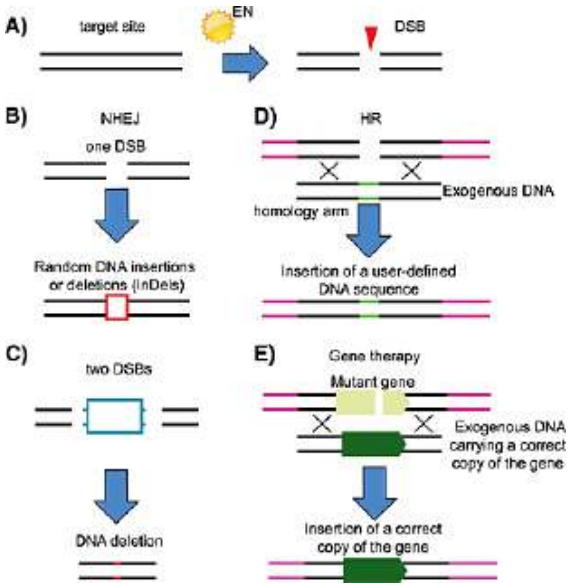

Figure 1. Genome Editing and DNA Repair Pathways[1]. A: Depending on the double-strand break (DSB) generation by a specific engineered nuclease, different scenarios can be observed during repair. B: A single DSB can be repaired by nonhomologous end joining (NHEJ) and could induce insertions or deletion (InDels) formation at the repaired site. C: In the case of a double DSB and a repair by NHEJ, a fragment of DNA could be deleted. D: If the DSB is repaired by HR in the presence of an exogenous DNA fragment containing sequence homology to the target site (homology arms), the break will induce the integration of the user-defined sequence into the targeted site. E: The induction of a DSB repair by homologous recombination (HR) using an exogenous DNA sequence ..may be used to introduce a functional copy of a mutated gene resulting in the correction of the endogenous gene.

Meganucleases

The most well-studied family of meganucleases, also called homing endonucleases, are the LAGLIDADG proteins. They are found in all kingdoms, and their natural function has yet to be elucidated, although they are classified as "selfish genetic elements" that function as RNA maturases, which facilitate the splitting of their own intron and/or function as highly specific endonucleases capable of recognizing and cleaving the exon-exon junction sequence where their intron is located[8]. These proteins recognize DNA sequences of 14 – 40 bp which it is able to then cleave; thus, they possess an extremely high degree of specificity. Meganucleases may be engineered to recognize specific sequences by making changes in their amino acid sequence, or by fusing protein domains from different enzymes. Fusing meganucleases with zinc fingers (ZFs) or Transcription Activator-Like Effector (TALEs) (described below) allows for the creation of novel enzymes with the binding affinity of ZFs and TALEs combined with the cleavage specificity of meganucleases[9].

Companies utilizing meganucleases for gene editing include Cellectis, Pregenen, and Precision Biosciences.

Zinc Finger Nucleases (ZFNs)

ZFNs were first published in a 1991 paper by Pavletich and Pabo in Science[10]. The Cys2-His2 zinc finger domain is one of the most common types of DNA-binding motifs found in eukaryotes. Engineered ZFNs work by utilizing multiple DNA-binding domains which contain amino acids able to recognize three bps (bps) per zinc-finger. The development of artificial zinc-finger proteins containing more than three naturally occurring zinc-finger domains allowed for recognition of 9 - 18 base pairs. This allowed for specificity within 68 billion bp of DNA – enough to target genes in the human genome for the first time[6]. The DNA-binding modules may be combined with an effector domain such as a nuclease to alter gene structure and function. For over ten years, ZFNs were the primary tool used for gene editing. However, limitations such as the inability to target certain nucleotide triplets and issues with specificity have hindered its use. Furthermore, engineered ZFs must be constructed in large oligo-

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nucleotide pool arrays which are expensive, labor intensive, and are not guaranteed to be specific to the intended sequence[11]. To date, ZFNs have been used to correct disease-causing mutations associated with X-linked severe combined immune deficiency, hemophilia, sickle-cell disease, and α1-antitrypsin deficiency, as well as disease-associated mutations within the SNCA gene in patient-derived human iPS cells[6].

Sangamo Biosciences, in conjunction with Sigma-Aldrich, have developed a proprietary platform (CompoZr) for zinc-finger construction, with thousands of proteins readily available for use to create ZFNs [6]. Sangamo has also developed ZFNs for therapeutic applications which are currently in clinical trials.

Transcription Activator-Like Effector-based Nucleases (TALENs)

In 2009, TALE proteins were discovered and published in the journal Science[12, 13]. TALEs are naturally occurring proteins found in the Xanthomonas bacteria and contain DNA-binding domains composed of 33 – 35 amino acid repeat domains, each of which have the ability to recognize a single bp. This allows for greater flexibility in targeting genes as compared to the three bp sequences recognized by ZF DNA binding domains. Many effector domains, including nucleases, are now available and can be fused with TALE proteins to create TALENs which may be utilized for targeted genetic modifications. One limitation of TALENs is that targeted sequences must begin with a T[6]. Furthermore, TALEs pose a challenge for assembly, sequence verification, and genetic stability due to their highly repetitive nature – even with an automated approach, constructing a TALE library for every protein coding locus in the human genome would take >$1M and 200d[14].

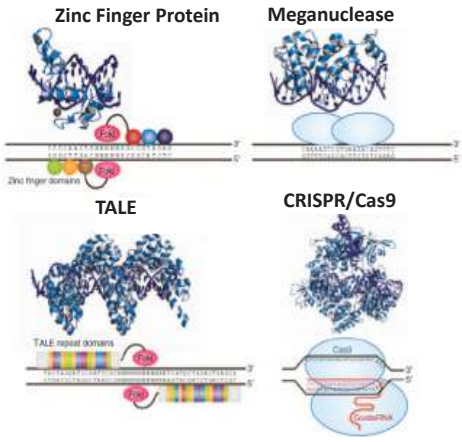

Figure 2: Common DNA Targeting Nuclease-Based Platforms for Genome Editing

Many custom TALE arrays are available through Cellectis Bioresearch, Transposagen Biopharmaceuticals, and Life Technologies. Cellectis has developed TALENs for therapeutic applications as well.

CRISPR/Cas9

Gene Editing

The ability of the CRISPR/Cas9 to cut specific DNA sequences was first shown in 2012, and its simplicity and ease-of-use compared to older gene editing technologies quickly generated great excitement in the scientific and medical world[15]. The ZFN and

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TALEN technologies require tedious protein engineering and optimization to create the DNA recognition proteins for each target DNA sequence, limiting their usage for the large-scale genomic manipulation required in order to bring products to market. Unlike the ZFN and TALEN systems which utilize protein binding domains, the CRISPR/Cas9 system utilizes a simpler RNA-guided mechanism for introducing precise mutations at a specific target[16].

Bacteria and archaea have evolved a natural CRISPR/Cas system which recognizes and eliminates foreign DNA by RNA-DNA base pairing and subsequent cleavage of foreign DNA by the Cas proteins. There are three different types of CRISPR systems. Type I systems have a unique Cas3 protein which possesses both helicase and DNAse domains. Type III CRISPR/Cas systems possess a unique Cas10 protein which has an unknown function, but is known to be able to target both DNA and/or RNA [17]. The Type II system is the simplest among the three; its endonuclease activities are found in a single multidomain protein, Cas9, which is guided by a site-specific dual

RNA molecule, which can be easily modified (as will be discussed below). This simplicity and ease-of-programming makes Type II systems a very valuable tool for genomic engineering.

The Type II CRISPR/Cas9 system found in Streptococcus thermophiles is a highly modifiable nuclease tool for modifying DNA, encoded by a set of Cas genes and a set of CRISPR RNA (crRNA) genes[18]. The Type II CRISPR system incorporates foreign DNA sequences between repeat CRISPR sequences in the host genome which are transcribed into crRNA containing the foreign DNA sequence, known as the "protospacer" sequence. The crRNA forms a complex with transactivating CRISPR RNA (tracRNA), which then complexes with the Cas9 nuclease. The protospacer-encoding portion of the crRNA guides Cas9 to cleave the complementary foreign/target DNA sequence if they are adjacent to short sequences of DNA known as "protospacer adjacent motifs" (PAMs)[15]. Engineered CRISPR/Cas9 systems are composed of the Cas9 nuclease which is guided by a designed, chimeric single guide RNA (sgRNA). The sgRNA is composed of a fusion of the crRNA and tracRNA, which contains a 20 bp binding sequence at the 5' end that may be customized to target a desired sequence. The Cas9 induced DSBs are then used to introduce NHEJ-mediated sequence-specific mutations or to stimulate HDR using either double stranded or single stranded DNA templates[15]. Multiple sgRNAs may be delivered simultaneously in order to edit several sites within the mammalian genome, unlike the meganuclease, TALEN, and ZFN systems. Furthermore, crRNAs may be easily modified to target specific sequences, allowing for a highly flexible and multiplexed system[16]. Currently, an sgRNA library targeting every protein coding locus in the human genome can be constructed within a few weeks using chip-based oligonucleotide synthesis for ~$5,000 [11], which is one of the reasons why the CRISPR/Cas9 system has become so widely adopted in addition to its efficiency, simplicity, ease-of-use, and programmability.

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Gene Regulation

Modulation of gene transcription is done by either repression or activation. A CRISPR/Cas9-based system known as CRISPRi has been developed to silence endogenous and/or reporter genes in mammalian cells. This system is created by fusing dCas9 (a nuclease-deactivated form of Cas9) to a transcription repressor domain. dCas9 is able to bind to the coding sequence of a gene or its promoter region and, through complimentary binding by an sgRNA, is able to block the elongating activity of the RNA polymerase or interferes with the binding of the RNA polymerase to the promoter sequence which subsequently represses transcription[16]. RNA interference (RNAi) has been the conventional method for repression of gene expression; however, its off-target effects have hampered its use. CRISPRi exhibits minimal off-target activity when utilizing well-designed sgRNAs, and is highly sensitive to mismatches between the target DNA and the sgRNA – even a single mismatch near the 3' end has been shown to significantly decrease the efficiency of CRISPRi[19]. However, recent studies have shown that this may not necessarily be accurate, especially in situations with a high level of Cas9 and sgRNAs[7].

Epigentic Modification

Modifying the epigenome is another route for modulating gene expression. Cas9 mediated epigenome editing may be accomplished by fusing proteins such as the histone-modifying protein p300 to Cas9, which allows for activation of gene expression when targeted to promoters or enhancers[20]. In another example, to repress gene expression, four copies of the mSin3 domain were fused to the dCas9 scaffold and showed repression efficacy in HEK293T cells[21]. Another method of gene repression via epigenetic modification using the CRISPR/Cas9 system is the use of histone demethylase LSD1 fused to a dCas9 ortholog (dNmCas9) which was able to remove activating H3K4 methylation marks, leading to gene repression. Despite these available methods, epigenetic modifications using demethylase or methyltransferase effectors fused to Cas9 have not yet been developed[11].

In Vivo Ex Vivo Genome Modification

Before discussing the applications of gene editing technology, it is beneficial to note the different methods by which the technology may be applied in order to achieve the desired outcome, as seen in Figure 3. Gene editing may be performed either in vivo or ex vivo, by directly targeting and modifying cells in the zygote or in adult animals, including humans, or by modifying cells in culture and subsequently transplanting them back into the patient or animal, respectively.

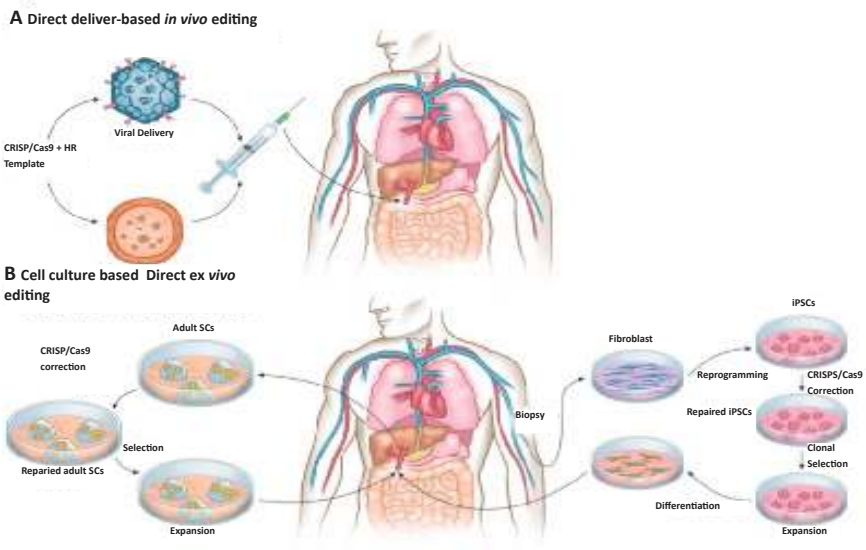

Figure 3. In Vivo and Ex Vivo Strategies for CRISPR/Cas9-Based Gene Therapies[2]. (A) In in vivo approaches, CRISPR/Cas9 components are directly delivered into the patient using either viral or nonviral vectors for in situ gene editing. (B) In ex vivo approaches, genes are edited in patient-derived cells. These can be generated by reprogramming (iPSCs) or direct expansion of somatic stem/progenitor cells, and are transplanted back into the same patient after the correction. CRISPR, clustered regularly interspaced short palindromic repeat; iPSCs (induced pluripotent stem cells).

In vivo gene editing involves direct or targeted delivery of nucleases to the diseased cells in the body. In vivo gene editing is advantageous over ex vivo gene editing in that it may be applied to diseases where the target cell population may not be manipulated ex vivo, and it is able to target multiple tissue types. In vivo gene editing is primarily performed using viral vectors which possess tissue-specific tropism, such as AAV (although AAV specificity and its use is thus generally limited to tissues such as the liver, muscle, and eye[22]). In addition, AAV has a limited carrying capacity which hinders its use with CRISPR/Cas9; the large amounts of virus required to achieve sufficient treatment efficacy may elicit an immune response, and the Cas9 nucleases may also elicit an immune response[16]. Targeting and controlling the amount of gene editing nucleases delivered to the target cell population also presents a challenge, as off-target effects remain a concern and may be difficult to predict. Targeted systems such as synthetic nanoparticles are currently being developed to address such challenges associated with viral vector-based delivery.

Ex vivo gene editing involves removing the target cell population from the body, modifying the genomes of the cells, and then transplanting the cells back into the original host. The primary advantage of ex vivo gene editing over in vivo gene editing is that it allows for a variety of delivery platforms to be utilized. Some examples of these delivery methods include electroporation, cationic lipids, cell penetrating peptides, and viral vectors. The efficacy of gene editing performed ex vivo is generally higher than that achieved in vivo, and ex vivo editing allows for better control of dosage as the number of cells reintroduced into the patient can be precisely controlled[22]. However, target cells often fail to survive ex vivo, or lose properties necessary for their in vivo functioning. This limits ex vivo gene editing to tissues that have adult stem cell populations which are amenable to culture and manipulation. In addition, cultured cells frequently poorly engraft back into the patient which decreases the effectiveness of the treatment[22].

Each method has their own benefits and drawbacks, and either in vivo or ex vivo methods for gene editing may be utilized for a variety of applications, including the creation of animal disease models for drug development or for therapeutic applications.

Applications of CRISPR/Cas9

The CRISPR/Cas9 system may be utilized for the creation of animal disease models which are crucial tools for understanding diseases and for drug development. Transgenic mouse models are traditionally created by HR in mouse embryonic stem cells, followed by injection of the cells into mouse blastocysts; however, this is a very time consuming and inefficient process. Furthermore, mammalian species other than mice are very difficult to use when creating chimeric animals due to the difficulty in culturing embryonic stem cells in vitro. The CRISPS/Cas9 system presents a more efficient and easier to-to-use method for generating animal

disease models. The CRISPR/Cas9 system is able to manipulate multiple genes in the germline or at the zygote stage in mice embryonic stem cells, and mRNA encoding Cas9 and sgRNAs can be directly injected into fertilized mouse eggs, thus avoiding the difficulties associated with ex vivo culture and producing mice with biallelic mutations in one or more genes[23]. The CRISPR/Cas9 system may also be used for HDR-mediated genome editing in embryos[24]. While in vivo delivery of Cas9 is challenging due to its large size, researchers have, however, been able to hydrodynamically inject a plasmid encoding Cas9 and sgRNAs to create a liver cancer mouse model[25]. Other organisms such as zebrafish, rat, sheep, goat, rabbit, pig, and monkeys have also been engineered to be transgenic models for a variety of genetic diseases[16].

CRISPR/Cas9 also offers therapeutic potential for curing genetic diseases and eliminating viruses in the genome to treat infectious diseases. In one such example, Long et al. were able to rescue mice with Duchenne muscular dystrophy (DMD) by correcting the Dmd gene mutation using HDR, partially correcting the DMD phenotype[26]. Bacterial and viral infectious disease may also be a target for treatment by CRISPR/Cas9; by targeting bacterial or viral genomic DNA with the sgRNA, the CRISPR/Cas9 system can introduce mutations to inactivate the genes or remove or replace those genes. HIV is known to integrate into the human genome in a latent state and represents a potential therapeutic target for CRISPR/Cas9 therapy via elimination of the latent HIV DNA. It is also worth noting that the CRISPR/Cas9 system is able to target viral covalently closed circular DNA in replicating cells and chronically infected hepatocytes, and thus has potential for treating acute and chronic hepatitis B infection[16]. There has also been work in improving the delivery of CRISPR/Cas9; Yin et al. developed lipid nanoparticles containing Cas9 mRNA with AAVs encoding an sgRNA and a repair template to correct an Fah-splicing mutation[27]. The CRISPR/Cas9 system may also be used for cell therapy, e.g. by modifying human stem cells to correct for mutations, after which the stem cells may then be grown and transplanted back into the patient.

Cell Therapy and CAR-T Cells

Cell therapy and gene therapy are both methods for treating diseases; however, they are significantly different. Cell therapy is the infusion or transplantation of whole cells in to a patient to treat a disease, whereas gene therapy treats disease by modifying the expression of genes or corrects abnormal genes. It should be noted that cell therapies may utilize gene therapy/gene editing, e.g. the use of gene editing to modify T-cells to express a chimeric antigen receptor (CAR) which recognizes specific cancer cell epitopes expressed on their surface, after which the modified T-cells (CAR-T cells) are reintroduced into the patient.

T-cells are immune cells which are able to kill foreign and cancer cells. Recently, they have been engineered to express a CAR on their surface which allows them to recognize and kill specific cells such as cancer cells that express a certain epitope on their surface. Gene editing methods such as the CRISPR/Cas9 system may be utilized to engineer these CAR-T cells which are then used for cancer immunotherapy[9]. Each of the different approach for gene editing have their benefits and drawbacks when it comes to CAR-T cells. Janssen plans on using the CRISPR/Cas9 system with CAR-T cells to knockout many HLA genes in order to avoid graft-vs-host disease (GvHD). Cellectis plans on using TALENs to knockout just one gene for the T-cell receptor (TCR) alpha chain (the TCR may interact with the CAR and reduce specificity[9]). In this context, Cellectis claims that TALENs are more efficient than CRISPR/Cas9, and their use does not result in the loss of T-cell expansion due to off-target effects, unlike when using the CRISPR/Cas9 system.

Gene Editing Therapies in Development

Because CRISPR/Cas9 has only recently been discovered, it is in early stage development. In an exciting and tight race between multiple biotech companies vying to be the first to use CRISPR/Cas9 in humans in a clinical trial, the Chinese company Chengdu MedGenCell has emerged as the first to do so in a clinical trial (NCT02793856) set to begin in August of 2016 using ex vivo production of PD-1 knockout T-cells for the treatment of non-small cell lung cancer. Meanwhile, in June 2016, the NIH approved a proposal by the University of Pennsylvania to use CRISPR/Cas9 to engineer CAR-T cells for the treatment of three types of cancers. It should be noted, however, that the purpose of the trial is to determine the safety of

using the CRISPR/Cas9 system in humans rather than to evaluate therapeutic efficacy; the clinical trial is expected to begin this year. Editas plans to begin in 2017 a clinical trial to treat an orphan disease, Leber congenital amaurosis (LCA), using their CRISPR/Cas9 system.

While CRISPR/Cas9 has only recently started to be developed for use in humans, the other types of engineered nucleases have already made progress in the clinic. Sangamo BioSciences currently has multiple clinical trials using ZFNs to treat gliomas and HIV. In 2015, Cellectis successfully treated a leukemia patient using one of their TALEN-engineered UCART products.

Organism	Disease type and application	Strategy
Zebrafish	Pfeiffer Syndrome	HDR induces Pro252Arg gain of function
Xenopus	Albinism	NHEJ-induced simultaneous disruption of two X. laevis tyrosinase homeologs
Mouse	Liver and lung carcinoma	p53,pTEN,KRAS and β-catenin
	Pancreatic cancer	Lkb1 deletion and Kras modification
	Medulloblastoma and glioblastoma	Somatic gene modification of Ptch1 or multiple genes (p53,pTEN,Nf1)
	Genome-wide screening for tumor genes	Knock-in mice with gRNA are used to screen oncogenes
	Rett syndrome	Generation of MeCP2-dificient mice
	Cataracts	HDR-irduced correction of mutant Crygc gene
	Acute myeloid leukemia	Lentivirus-delivered sgRNA: Cas9 is used to modify several cancer driver genes together
	HBV	Cleave HBV DNA
	Alveolar Rhabdomyosarcoma	Pax3-Foxo1 chromosome translocation
Rat	Ducherne muscular dystrophy (DMD)	Edit two exons in the rat Dmd gene
Rabbit	Tyrosinase disorders	Zygote direct injection of Cas9 and sgRNA to tyrosinase gene
Pig	Gene-modified pigs	One-stage-embryo injection of Cas9/sgRNA
	Generation of B Cell-Deficient Pigs	IgM-targeting Cas9 delivery to produce B cell-deficient mutant pigs by somatic cell nuclear transfer (SCNT) technology
Monkey	Gene-modified cynomolgus monkey	Coinjection of one-cell embryos with Cas9 mRNA and sgRNAs
	Human AHC-HH	Dax1-null mutations
	Ducherne muscular dystrophy (DMD)	Monkey dystrophin gene disruption

Table 1. CRISPR/Cas9- mediated generation of animal models and applications in human health

Key Companies in the Gene Editing Space

Editas

Editas is one of the leading companies in the gene editing space, with expertise in CRISPR/Cas9 and TALENs technologies. Editas is working on translating its genome editing technology into novel human therapeutics, focusing on engineering a modified version of Cas9 which possesses better target specificity. The company was founded by five leaders in the field and raised $43M in a series A round of funding, led by Flagship Ventures, Polaris Partners, and Third Rock Ventures. To-date, they have raised $163M from private investors. Editas currently has exclusive rights to the one issued patent for CRISPR, and they have secured key exclusive IP agreements with Massachusetts General Hospital and Duke University for crucial CRISPR technologies. They plan on entering clinical trials in 2017 to treat Leber congenital amaurosis (LCA). Editas recently had an IPO in February of 2016, raising $94M.

Caribou Biosciences

Caribou was founded out of UC Berkeley by one of the cofounders of Editas, Jennifer Doudna. Doudna broke off from Editas when the key CRISPR patent was given to another Editas founder, Feng Zhang. Doudna filed a separate patent application for the CRISPR technology which is currently pending. Editas and Caribou are currently in a patent dispute for the CRISPR technology, the result of which will dramatically impact both companies. Caribou is working on applying their technology for therapeutics, agriculture, research, and industrial biotechnology. Dr. Rodolphe Barrangou at NC State University is collaborating with Caribou to understand the DNA sequence targeting mechanism of the CRISPR/Cas9 system. Caribou is backed by an $11M series A funding round from Atlas Venture and $30M in a series B round of funding.

Intellia

Intellia was founded by Caribou Biosciences and Atlas Venture in 2014, and is funded by Atlas Venture and Novartis Institutes for Biomedical Research. They are working on improving the sgRNA used in their CRISPR/Cas9 system which targets the desired DNA sequence. Caribou outlicensed their CRISPR technology to Intellia in return for exclusive license rights for all human therapeutic applications, while Novartis has retained exclusive rights to use Intellia's CRISPR platform for development of CAR-T cell therapies, as well as non-exclusive rights for limited in vivo therapeutic applications of CRISPR and a 5-year collaboration with Intellia for which they will provide an initial payment, technology access fees, and funding. Intellia recently raised $108M in an IPO in May of 2016.

Bayer acquired a majority stake in CRISPR Therapeutics and also agreed to provide a minimum of $300M over the next five years for R&D efforts, developing the CRISPR/Cas9 technology to treat conditions such as blood disorders, blindness, and heart disease.

Cellectis

Cellectis is a well-established French company founded in 1999 and is involved in both gene editing and cancer immunotherapy. In 2004, Cellectis partnered with BASF Plant Science to develop meganucleases for agricultural and nutritional applications. Cellectis partnered with Pregenen in 2011 to develop meganucleases. The company has worldwide rights to a patent family entitled "Engineering Plant Genomes Using CRISPR/Cas Systems" and an exclusive license for the commercial application of

ID	Sponsor	Phase	Title	Status (as of July 2016)
NCT00842634	Sangamo	1	Autologous T Cells Genetically Modified at the CCR5 Gene by Zinc Finger Nucleases SB-728 for HIV	Completed
NCT01044654	Sangamo	1	Phase 1 Dose Escalation Study of Autologous T Cells Genetically Modified at the CCR5 Gene by Zinc Finger Nucleases in HIV-Infected Patients	Completed
NCT01082926	City of Hope Medical Center	1	Phase I Study of Cellular Immunotherapy for Recurrent/Refractory Malignant Glioma Using Intratumoral Infusions of GRm13Z40-2, An Allogeneic CD8+ Cytolitic T Cell Line Genetically Modified to Express the IL 13-Zetakine and HyTK and to be Resistant to Glucocorticoids, in Combination With Interleukin-2	Completed
NCT01252641	Sangamo	1/2	Study of Autologous T Cells Genetically Modified at the CCR5 Gene by Zinc Finger Nucleases in IV Infected Subjects	Completed
NCT02225665	Sangamo	1/2	Repeat Doses of SB-728mR-T After Cyclophosphamide Conditioning in HIV-Infected Subjects on HAART	Active
NCT01543152	Sangamo	1/2	Dose Escalation Study of Cyclophosphamide in HIV-Infected Subjects on HAART Receiving SB-728-T	Active
NCT02500849	Sangamo	1	Safety Study of Zinc Finger Nuclease CCR5-modified Hematopoietic Stem/Progenitor Cells in HIV-1 Infected Patients	Recruiting
NCT02808442	Cellectis	1	Study of UCART19 in Pediatric Patients With Relapsed/Refractory B Acute Lymphoblastic Leukemia (PALL)	Recruiting

Table 2. Representative Ongoing and Completed Gene-Editing Clinical Trials[8].

CRISPR Therapeutics

CRISPR Therapeutics was founded in April of 2014 by Emmanuelle Charpentier, another one of the co-inventors of the CRISPR/Cas9 technology and who is also a part of Doudna's CRISPR patent. CRISPR Therapeutics is focusing on developing an algorithm which can reliably predict areas of potential CRISPR/Cas9 off-target activity. They have raised a total of $89M in Series A and B rounds and are backed by SR One, Celgene, New Enterprise Associates, Abingworth, and Versant Ventures. In December of 2015, TALEN technology from the University of Minnesota. They have five universal chimeric antigen receptor T-cell (UCART) products targeting specific cancers in various stages of clinical development. In November of 2015, Cellectis successfully treated a baby with acute lymphoblastic leukemia using its UCARTs. Cellectis offered an IPO in March of 2015, raising $228M.

Precision Biosciences

Precision has a ARCUS genome editing platform technology utilizing meganucleases which they plan on using for oncology, genetic diseases, and agriculture. Precision is developing meganucleases for engineering CAR-T cells. They possess a growing patent estate of over fifteen genome engineering patents in the US and Australia. Precision has filed suits against Cellectis in the US which are currently pending in the US District Court for the District of Delaware. One of their patents was recently challenged and upheld. They raised $26M in series A funding in May of 2015, and in February of 2016 entered into a collaboration with Baxalta to develop a series of allogeneic CAR-T cell therapies for multiple cancer indications.

Sangamo Biosciences

Sangamo is a well-established company using a gene editing system based on ZFNs. They have entered multiple Phase 1 trials, including Phase I trials for an HIV cure (NCT01252641, NCT00842634, and NCT01044654)[28]. The FDA approved their IND for SB-913 in June of 2016, a ZFN designed to treat Hunter syndrome (Mucopolysaccharidosis Type II). Sangamo has a partnership with Shire International GmbH to develop therapies for Huntington's disease, and a collaboration with Biogen to develop treatments for hemoglobinopathies, beta-thalassemia and sickle cell disease. They have outlicensed applications in high value laboratory research reagents, transgenic animals and commercial production cell lines to Sigma-Aldrich Corporation, and in plant agriculture to Dow AgroSciences. Sangamo raised $49M in an April 2000 IPO.

CRISPR/Cas9 Intellectual Property Issues[29, 30]

Currently, there is an ongoing legal battle over the issue of who owns the rights to the CRISPR system. Jennifer Doudna at UC Berkeley is in dispute with Feng Zhang at the Broad Institute and MIT over who owns the rights to fundamental aspects of the CRISPR technology. Doudna filed a patent (No. 13/842,859) on March 15, 2013, one day before the US Patent and Trademark Office (PTO) enacted the first-to-file rules. Her patent application laid out 155 broad claims to the CRISPR technology, and was given a priority date of May 25, 2012. On October 15, 2013, Zhang filed a patent for CRISPR (No. 14/054,414) – months after the first-to-file rules had come into effect, but he claimed a priority date of Dec. 12, 2012 which is under the old first-to-invent rules. Zhang also filed an Accelerated Examination Request which was accepted by the PTO, resulting in the issue to Zhang of his first CRISPR patent in April 15, 2014.

Doudna's attorneys amended her patent application multiple times, but an unknown third party submitted reports of prior art in order to stop her application from being accepted. On April 13, 2015, Doudna's attorneys sent in another amended application which cancelled all previous claims and replaced them with 82 new claims, as well as a Suggestion of Interference which claimed that 10 of Zhang's issued patents were interfering with their patent application. On Dec. 21, 2015, the examiner corps recommended to the Patent Trial and Appeal Board (PTAB) that it initiate an interference proceeding, and changed the status of Doudna's patent application as such. This is significant because it, at the least, discourages a settlement between the parties. The interference proceeding began with an interlocutory phase on March 9, 2016, where Doudna and Zhang presented briefs and the PTAB considered motions regarding why each party is entitled to certain priority dates. The onus lies on Zhang, as the junior party, to present evidence showing that he was the first-to-invent. After the interlocutory phase of the interference proceeding, the panel may decide who will win the patent, or, move the proceeding to the testimonial phase if there is conflicting evidence, after which the patent awardee will be decided. Currently, Doudna's attorneys are claiming that Zhang "never had or made use of" tracrRNA, a crucial component of the CRISPR/Cas9 system[29]. Zhang's attorneys deny these claims. Doudna's attorneys say they are open to a settlement, but there have not been any formal discussions between the two parties. As such, the dispute looks to be heading into the next phase, a hearing which may be scheduled in November, 2016. It should be noted that, after the issue is decided by the PTAB, the losing party may file an appeal, which would draw out this patent dispute for years.

CRISPR/Cas9 Challenges

In addition to the battle over IP outlined above, the CRISPR/Cas9 technology faces some difficulties before its use may be translated into the clinic. Some limits of the CRISPR/Cas9 system include its off-target effects, difficulties associated with delivery to target cells, and the potential for an adverse immune response to the bacterial Cas9 protein, as well as regulatory and ethical hurdles and issues with reimbursement which must be addressed.

Off-Target Effects and Specificity

There are many research efforts currently underway aiming to increase the specificity of the CRISPR/Cas9 system. One strategy is to engineer the Cas9 protein into a nickase protein which facilitates HDR, minimizes mutagenic activity, and has higher specificity[31]. Furthermore, multiple sgRNAs can be created for each gene in order to reduce off-target effects[15]. Finding highly unique target sequences via computational approaches may also improve the specificity. Modification of the 20 bp sgRNA to be 17 bp was found to increase targeting specificity because the truncated sgRNAs possess a reduced binding affinity with the target sequence and are thus more sensitive to mismatches[15]. There has also been research into the production of Cas9 variants with alanine substitutions at residues predicted to be involved in stabilizing the target DNA strands to increase specificity. Using this strategy, Slaymaker et al.[32] produced a Cas9 variant which possessed decreased off-target indel formation while preserving on-target activity, and Kleinstiver et al.[33] used a similar method to produce a Cas9 variant that retained high on-target activity with minimal off-target activity. It should be noted that improved methods must be developed for determining off-target effects of targeted nuclease systems, current methods are unable to identify sites cleaved at low frequencies in a bulk cell population or cannot predict nuclease activity at the identified off-target sites[8].

Delivery

Delivery remains a significant challenge for the gene editing and gene therapy field, including the CRISPR/Cas9 system which has its own unique challenges. In vivo editing poses the additional challenges of cell or tissue type specificity, immunogenicity, and biocompatibility. There are two broad types of delivery methods which may be used for the delivery of CRISPR/Cas9: viral or non-viral.

Viral vectors such as lentiviral and adeno-associated virus (AAV) vectors may be used with the CRISPR/Cas9 system. They are able to provide transient nuclease expression and lentiviral vectors have sufficient capacity to encode multiple nucleases or sgRNA expression cassettes[8]. Lentiviral vectors are able to transduce both dividing and non-dividing cells, and have a large carrying capacity of 10 kb, allowing for packaging of Cas9 with four sgRNAs[34, 35]. They have also shown to be highly efficient at transducing T-cells and hematopoetic stem cells (HSCs)[8]. However, lentiviral vectors are expensive to produce and have a risk of insertional mutagenesis. Adeno-associated virus (AAV) has low immunogenicity, and has been shown to be useful for targeting certain tissue types such as the liver, eye, nervous system, and skeletal and cardiac muscle in both preclinical models and clinical trials[8]. However, AAV is constitutive, patients may have antibodies to AAV, and AAV has a limited carrying capacity of ~4.7 kb which limits its ability to carry Cas9 (as well as TALENs, however, ZFNs may be packaged into a single AAV). Engineering or employing a smaller Cas9 gene or using a smaller Cas9 ortholog may allow for delivery via AAV[8, 15].

One of the simplest non-viral methods is electroporation, which may be utilized for ex vivo delivery of plasmid DNA encoding CRISPR/Cas9 components. The direct delivery of nucleases or Cas9-sgRNA complexes by electroporation or fusion to cell-penetrating peptides (CPP) has been employed for delivery of CRISPR/Cas9[21]. Efforts have also been made into utilizing lipids and lipid-based nanoparticles such as liposomes for delivery[3]. Nanoparticle and lipid-based in vivo mRNA and protein delivery systems offer an alternative strategy and possess transient delivery which is beneficial in certain cases. Furthermore, the surface of nanoparticles may be functionalized to improve circulation time, add targeting ability, or increase uptake[35]. Hydrodynamic injection may also be used for in vivo delivery, but only to the liver, and it is not clinically applicable, although it may be used for the creation of mouse models such as a mouse model of liver cancer[34].

Immunogenicity

It should be noted that exposure to these nucleases may provoke an immune response, especially for TALENs and Cas9 which are derived from microbial organisms. One strategy to address this challenge is to humanize certain peptide fragments, which may reduce immunogenicity[6].

Regulatory Considerations [36]

While some genetically modified crops have avoided regulatory hurdles, human gene therapy and gene editing will not be able to avoid a litany of regulatory requirements. Researchers must be prepared to deal with rigorous and complex regulatory oversight in all aspects of gene therapy/editing clinical trials, from pre-clinical efficacy and toxicology to product manufacturing to clinical trials design. There are currently no approved human gene therapy products available on the market, although there are some approved cell therapies. A comprehensive review of the regulatory requirements for gene editing and gene therapy is out of the scope of this report, a brief overview is provided below.

The FDA Center for Biologics Evaluation and Research (FDA - CBER) regulates cell therapy products, gene therapy products, and certain devices related to cell and gene therapy. All gene therapy products and most somatic cell therapy products are regulated by the FDA in 62 CFR 9721. Quality control of the manufacturing process must be strictly maintained, the FDA regulations may be found in 21 CFR 210, 211, 312, and 600. The collection and/or culture of cells must follow guidelines, and the potency, viability, contaminants, purity, general safety must all be assessed. Vectors must be carefully constructed and characterized, with lot-to-lot release testing of sterility, identity, potency, purity, and safety.

Gene therapy clinical trials are governed by a wide range of legislation and guidelines. Legislation primarily serves to protect the human subject(s), the general public, and the environment. Regulatory institutes in this area include the Recombinant DNA Advisory Committee (RAC), the FDA Center for Biologics Evaluation and Research (FDA - CBER), the European Medicines Agency (EMA), and the Gene Therapy Discussion Group of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). It should be noted that the FDA has jurisdiction in gene therapy clinical trials, regardless of the funding source or research site[37]. The FDA CBER website provides information regarding their requirements (http://www.fda.gov.libproxy.lib.unc.edu/BiologicsBloodVaccines/CellularGeneTherapyProducts/default.htm), including information regarding IND submission (http://www.fda.gov.libproxy.lib.unc.edu/BiologicsBloodVaccines/DevelopmentApprovalProcess/InvestigationalNewDrugINDorDeviceExemptionIDEProcess/default.htm). The Institutional Biosafety Committee (IBC), Cancer Center Scientific Review Board (NCI), GCRC Advisory Board (NCRR), and the FDA must all approve the clinical protocol. Good Clinical Practices (GCP) guidelines must be followed and maintained during the clinical trial, and studies must obtain IRB approval as well as informed consent.

Ethical Considerations [38]

There are many ethical considerations for gene therapy which has considerable risks; a thorough review of all ethical issues surrounding gene therapy and gene editing are outside of the scope of this report, a summary is provided below. Some ethical considerations include determining what is “normal” and what is a “disability” that can be treated, or the ethical implications of germline gene therapy/editing. As such, the FDA has taken careful measures to address any possible ethical dilemmas. Scientists must seek approval by the FDA for their proposal which must follow strict guidelines set by the FDA, and they must report side effects during the course of the trial. Informed consent must be given in clinical trials; the requirements for informed consent may be found in 45 CFR 46 and 21 CFR 56[37]. At the International Summit on Human Gene Editing held in Dec. 2015 by the US Academies of Sciences and Medicine, the Royal Society, and the Chinese Academy of Sciences, a consensus regarding certain aspects of gene therapy was reached: basic research should be subject to appropriate legal and ethical rules and oversight, and no human embryos or germline cells which have undergone gene editing should be used to establish a pregnancy; somatic gene therapy may be evaluated within the existing and evolving regulatory framework for gene therapy; and (human) germline editing may not be performed.

Commercial Challenges

Most of the challenges discussed so far involve the complexities of sciences. However, one of the key business issues which will need to be addressed is reimbursement. Let's take a look at some real world examples. Biologics such as anti-TNF drugs which treat but do not cure the underlying disease, cost $30k - $50k per year. This is a significant cost when diseases such as RA can be detected in the 20s and 30s which means treatment can be 30 years or more. On the end of the spectrum, are curative therapies such Gilead's Harvoni for Hepatitis C (HCV). Prior to Harvoni, there was no cure and treatment options. Harvoni costs $85k (without discounts) and cures HCV for greater than 98% of patients. Yet there has been significant push back against Harvoni but very little against anti-TNF therapies. Thus it is likely that the transformative nature of gene editing will make pricing and reimbursement a difficult and intricate process.

Conclusion

Gene editing is a revolutionary tool which has recently received much publicity and investment with the advent of CRISPR/Cas9, the next step in gene editing. Although CRISPR/Cas9 has generated a great amount of excitement during the past two years, it has been over two decades since gene editing was introduced, and gene editing has yet to be utilized in the clinic. The technology must still mature significantly before it can be used in humans. Innovative breakthrough technologies such as gene editing/CRISPR/Cas9 or, as an analogy, RNAi, often go through cycles of receiving significant attention, excitement, and investment, followed by a period of maturation where the technology receives less attention from the public but slowly develops as research progresses and the technology is vetted. If the technology is to survive this process, pharmaceutical companies must form alliances to develop the technology together, as has been seen with the oncological therapy revolution over the last 15 years which started out with just a few companies and has now expanded to nearly all of the largest pharmaceutical companies having oncological therapies in their pipeline, often in a partnership with each other. Interpreting and being able to understand the intricacies of the technology and when/where to invest will be important for the growth of this technology. Furthermore, the IP controversy surrounding CRISPR/Cas9 must be resolved and the right therapeutic area(s) must be chosen, in addition to solving the technical barriers which exist (such as off-target effects, delivery, toxicity and immunogenicity, not to mention ethical concerns). However, if gene editing lives up to its potential, this still-nascent technology will revolutionize the world of science and medicine, bringing us another step closer to truly personalized medicine.

"Innovative breakthrough technologies often go through cycles of receiving significant attention, excitement, and investment, followed by a period of maturation where the technology receives less attention from the public but slowly develops as research progresses and the technology is vetted. If [gene editing] technology is to survive this process, pharmaceutical companies must form alliances to develop the technolog together, as has been seen with the oncological therapy revolution over the last 15 years."

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Authors

Myung Soo Kim, PhD
Analyst
Kineticos

Dr. Kim recently graduated from the Eshelman School of Pharmacy, Division of Molecular Pharmaceutics at UNC–Chapel Hill. His research involved a novel approach to formulating exosomes, nano-sized extracellular vesicles used in intracellular communication, to deliver poorly soluble anticancer drugs for the treatment of pulmonary metastases.

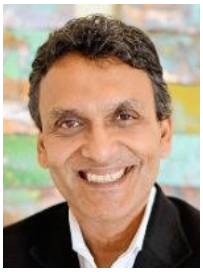
Prior to earning his PhD, Dr. Kim received a BS in Biology from UNC–Chapel Hill.

Mark Osternan
Senior Vice President
Kineticos

Mr. Osterman adds more than 25 years of experience in the biopharmaceutical industry to the team.

Mr. Osterman held roles in sales, clinical, and marketing at GlaxoSmithKline before moving to Johnson & Johnson, where he launched their stem cell internal venture and was responsible for the cardiovascular, pulmonary, metabolic, and mimetibody therapeutic areas in the Global Biologic Strategic Marketing.

Mr. Osterman earned a dual BA in Business Management and Economics from North Carolina State University.



Shailesh Maingi
Founder and CEO
Kineticos

Mr. Maingi founded Kineticos Life Sciences in 2012 and develops long-term and day-to-day execution strategies.

Mr. Maingi serves on the Board of Directors at Gallus Biopharmaceutical and he serves on the advisory boards of Novolipid and Enci Therapeutics.

Prior to founding Kineticos Life Sciences, Mr. Maingi was a senior executive at Cardinal Health/ PTS and Sigma-Aldrich/Supelco, where he held leadership roles in strategy, business development, and marketing.

Kineticos
LIFE SCIENCES CONSULTING

3005 Carrington Mill Blvd
Suite 510
Morrisville, NC 27560
Office: 919.678.3206
Email: Info@Kineticos.com

www.kineticos.com

About Kineticos
Kineticos is a specialized management consulting firm serving the life science industry. The firm is focused on identifying opportunities to drive strategic growth and achieve operational excellence for it clients.

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