CRISPR/Cas9: Transcending the Reality of Genome Editing

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With the expansion of the microbiology field of research, a new genome editing tool arises from the biology of bacteria that holds the promise of achieving precise modifications in the genome with a simplicity and versatility that surpasses previous genome editing methods. This new technique, commonly named CRISPR/Cas9, led to a rapid expansion of the biomedical field; more specifically, cancer characterization and modeling have benefitted greatly from the genome editing capabilities of CRISPR/Cas9. In this paper, we briefly summarize recent improvements in CRISPR/Cas9 design meant to overcome the limitations that have arisen from the nuclease activity of Cas9 and the influence of this technology in cancer research. In addition, we present challenges that might impede the clinical applicability of CRISPR/Cas9 for cancer therapy and highlight future directions for designing CRISPR/Cas9 delivery systems that might prove useful for cancer therapeutics.

The increasing burden of cancer in the human population represents a major concern for our society, and finding alternative treatments that are safe as well as efficient has become a major goal for researchers around the world. For the past decades, we have witnessed an effervescence of technologies that explore DNA structure and function, and our understanding of cancer has expanded to an extent that enables characterization of this disease at a deeper molecular level. The progression of basic research to reach clinical applications necessitates reliable pre-clinical models of cancer in which therapeutic strategies and agents can be evaluated for efficacy or efficiency.

Introducing targeted modifications in the genome for functional studies and cancer modeling or, moreover, for therapeutic purposes, requires highly efficient systems that are able to alter the existing DNA pattern with great precision. Nucleases of bacterial origin represent powerful tools that have been widely used for genome engineering purposes in an attempt to studying gene function and, ultimately, to implement new therapeutic strategies.

Among the existing nucleases with genome editing capabilities, the CRISPR system surpasses other nuclease-based systems, such as zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), in terms of simplicity of design and versatility1–3 (Table 1). By 2013, the ability of the CRISPR/Cas9 system to engineer mammalian cell genomes has been experimentally validated, and the crystal structure of the Cas9 effector complex was resolved in 2014.4 Among the three CRISPR/Cas systems (I–III) identified in both bacteria and archaea, the type II system from Streptococcus thermophilus or Streptococcus pyogenes is the most versatile for genome engineering purposes.5

The CRISPR/Cas9 type II system consists of the Cas9 nuclease and a single guide RNA (sgRNA or gRNA), which is a fusion of a CRISPR RNA (crRNA) and a trans-activating crRNA (tracrRNA) that binds Cas9 nuclease and directs it to a target sequence based on a complementary base-pairing rule. The target sequence must be adjacent to a protospacer-adjacent motif (PAM) consisting of a canonical NGG or NAG sequence. At the recognition site, a double-strand break (DSB) is generated that can be repaired by non-homologous end joining (NHEJ), resulting in small insertions or deletions (indels) usually associated with loss of function (knockdown/knockout) (Figure 1). In the presence of an exogenous donor DNA, by a homology-directed recombination (HDR) mechanism, precise modifications can be achieved at the targeted site, resulting in gain of function (knockin).6

However, Cas9 can tolerate, to a certain extent, mismatches between the sgRNA and the target sequence in the genome, resulting in off-target effects, as some previous studies have shown.7,8 These undesirable effects of the CRISPR/Cas9 system might impede the use of this genome editing technology for clinical applications; therefore, a great
Minimizing the Off-Target Effects of CRISPR/Cas9

Optimizing the sgRNA design represents one approach for reducing the off-target effects of CRISPR/Cas9. It has been shown that the base composition of the 5’ sequence of the sgRNA can have a profound effect on the efficiency of CRISPR/Cas9, and large-scale studies of sgRNA libraries led to design algorithms for maximizing the on-target activity and reducing off-target effects. Several online tools are now available to assist researchers in designing sgRNAs with a higher specificity for a desired genomic locus (Table 2).

The activity of Cas9 nuclease is another prime factor causing undesirable off-target effects of the CRISPR/Cas9 system; specifically, elevated levels of Cas9 has been associated with unspecific cleavage. Therefore, controlling the activity of Cas9 nuclease would lead to a significant reduction of unwanted “side effects.” In this regard, several approaches that use chemical or physical agents to achieve conditional expression of Cas9 have been under investigation by researchers and are illustrated in Figure 2.

Turning the activity of Cas9 on and off confers to the researcher a means for a temporal control, limiting the exposure time of the genome to Cas9 nuclease, thereby reducing the off-target effects the CRISPR system exhibits under normal conditions. Placing Cas9 under the control of a tetracycline-responsive element (TRE) promoter confers the possibility to achieve a conditional expression in the presence of tetracycline/doxycycline (Tet/Dox). However, even in the off state, when Tet/Dox are absent, Cas9 expression still exhibits “leakiness,” as recently reported.14 Placing the sgRNA under control of a hybrid Tet-responsive promoter proved to be a more advantageous alternative to obtain a tighter control of the off-target cleavage in a Dox-dependent manner.14 Regardless whether Cas9 or sgRNA expression is regulated in a Dox-dependent manner, such inducible systems require additional trans-acting factors and promoters to be incorporated in the construction of the delivery system, which, in the case of adenovirus vectors, is quite problematic because of their limited cloning capacity. Therefore, inducible CRISPR/Cas9 systems that use minimal genetic elements to achieve conditional expression of Cas9 might prove to be useful in designing delivery vectors for therapeutic applications.

In one study, researchers rendered Cas9 nuclease activity dependent on 4-hydroxytamoxifen (4-HT) by fusing a hormone-binding domain of the estrogen receptor (ERT2) to Cas9. This fusion ensures sequestration of Cas9 nuclease to the cytoplasm in the absence of 4-HT, consequently limiting the activity of the enzyme. A nuclear import is seen within the first 6 hr after induction with 4-HT, and optimal nuclease activity occurs at a time interval of 4–8 hr at 1 μM 4-HT; therefore, the off-target effects are reduced to a minimum. In a similar manner, Davis et al.13 used the self-splicing properties of inteins to render Cas9 nuclease active in the presence of 4-HT. In this report, the ERT2 domain is fused to intein to render

| Genome Editing Tool | ZFNs | TALENs | CRISPR/Cas9 |
|---------------------|------|--------|-------------|
| Features            | ZF-FokI nuclease protein fusion | TALE-FokI nuclease protein fusion | Cas9 nuclease and sgRNA |
| Target site identification | protein-DNA interaction, capable of targeting virtually any site | protein-DNA interaction, capable of targeting virtually any site | RNA-DNA interaction, site selection restricted by the NGG motif, which occurs statistically every 8 nt in a random DNA sequence |
| Genome altering | DSBs directed by the FokI domain, repaired through NHEJ or HDR | DSBs directed by the FokI domain, repaired through NHEJ or HDR | DSBs directed by Cas9, repaired through NHEJ or HDR |
| Design | ++ | ++ | +++ |
| Efficiency | ++ | ++ | +++ |
| Biallelic targeting | ++ | ++ | +++ |
| Off-target effects | ++ | more specific than ZFN | low compared with ZFN and TALEN as a result of allowed mismatches by the Cas9 nuclease between sgRNA and the DNA target sequence |
| Multiplexing | + | + | +++ |
| Size and delivery | the ZFN monomer is significantly smaller than Cas9 | TALEN monomers are situated in the middle of Cas9 and ZFN nucleases in terms of size | massive Cas9 protein encoded by a 4.2-kb sequence |

A deal of effort has been made to improve the efficiency and specificity of CRISPR/Cas9.

In this review, we summarize recent improvements made in CRISPR/Cas9 design and flexibility and the applications of this genome editing technology for cancer research. At the end of the manuscript, we discuss challenges and future perspectives of CRISPR/Cas9 in cancer therapy.
Table 2. Online sgRNA Design Tools

| Platform         | Platform Link                                                                 | Reference |
|------------------|-------------------------------------------------------------------------------|-----------|
| CRISPR Design    | http://crispr.mit.edu/                                                        | 9         |
| E-CRISP          | http://www.e-crisp.org/E-CRISP/                                               |           |
| CRISPR MultiTargeter | http://www.multicrispr.net/basic_input.html                                    | 88        |
| sgRNA Designer: CRISPRko | http://portals.broadinstitute.org/grrp/public/analysis-tools/sgrna-design    | 10, 89    |
| Off-Spoter       | https://cm.jefferson.edu/Off-Spoter/                                          | 90        |
| CCTop            | http://crispr.cos.uni-heidelberg.de/index.html                                | 91        |
| CHOPCHOP         | http://chopchop.cbcb.ubc.ca/index.php                                         | 92, 93    |

Figure 1. CRISPR/Cas9 Mechanism of Action

The original bacterial CRISPR/Cas9 design has been translated into an engineered instrument for genome editing purposes and is capable of introducing specific modifications in the target cell. In this regard, the vector comprising the crRNA and tracrRNA that together constitute the RNA molecule for Cas9 guidance (gRNA) is introduced in the desired cell, where it passes the cytoplasmic milieu toward the nucleus. After delivery to the nucleus, the Cas9 gene encoded by the experimental vector is transcribed and exported into the cytoplasm for translation of Cas9 nuclease. After synthesis of the active protein, the gRNA, transcribed by its own promoter, interacts with the Cas9 nuclease, resulting in the ribonucleic-protein effector complex that is internalized back into the nucleus. The cleavage of the double-stranded genomic DNA takes place in a guided manner, where the crRNA sequence of gRNA directs Cas9 toward the specific locus, based on sequence complementarity, which is positioned adjacent to the PAM. When cleaved, the continuity of the host DNA can be restored through NHEJ, where the hanging ends join together, creating small indels, or through HDR in the presence of a donor DNA.

Other models have used the propriety of light-inducible heterodimerization proteins for the modulation of Cas9 activity. These “light approaches” use a catalytically inactive form of Cas9 or a split Cas9 variant that is fused to light-responsive proteins.22 Upon stimulation with blue light, Cas9 becomes active either by transitioning from an inactive form19 or by reconstitution of the whole active Cas9 nuclelease from the C domain and N domain of the enzyme.20 Although such systems are to some extent reversible and adjustable for regulating Cas9 activity, they are limited to in vitro applications, under which blue light is relatively easy to apply. For in vivo applications, stimulating Cas9 activity with blue light requires more invasive and dedicated equipment to achieve an efficient effect, a fact that might limit the use of such systems in clinical contexts.

A recent study elegantly used a self-restricted CRISPR/Cas9 system to achieve control of Cas9 nuclelease to minimize off-target effects.21 In addition to a specific targeting sgRNA, the authors co-expressed a catalytically inactive form of Cas919 or a split Cas9

As reviewed above, limiting undesired off-target effects is not an easy task, requiring additional genes and inducers, modifications to Cas9, or even invasive approaches to obtain spatiotemporal control of CRISPR/Cas9 so that this technology can move forward to therapeutic applications in a clinical context. In regard to “simple is beautiful,” we definitely need an easy and, at the same time, reliable system that will give us the possibility to engineer our “faulty” genomes to a healthy state and, furthermore, to an improved state of well-being.

Multiplexing CRISPR/Cas9 Editing Capabilities

Despite the fact that we currently might have a simple CRISPR/Cas9 system that is, to some extent, safe and efficient in targeting a specific genome site, there are still many other obstacles to overcome to successfully implement this strategy in clinical therapy. In some...
instances, targeting a single site in the genome is not sufficient to achieve a full therapeutic effect because some diseases, like cancer, have a multigenic basis and require targeting whole gene networks that sustain the pathological state of the cell. Therefore, such an approach would require the use of multiple sgRNAs able to target multiple genomic loci, adding an extra level of complexity for designing CRISPR/Cas9 systems.

Traditionally, this would mean that one must have at least two sgRNA expression cassettes on the same construct or placed on two different constructs. Co-transfection of two vectors limits the efficiency of the CRISPR/Cas9 system in vitro and, furthermore, in vivo. In addition, having multiple sgRNA expression cassettes on the same construct poses a limit of physical constraints in the number of sgRNA cassettes because of their limited cloning capacity, as seen for AAV vectors.15

Indeed, multiplexing CRISPR/Cas9 editing capabilities would offer several advantages over a conventional single sgRNA-based system for targeting multiple sites and gene networks. To date, researchers have developed two strategies for this approach. As mentioned above, one approach uses multiple sgRNA expression cassettes made of an individual RNA polymerase III promoter, sgRNA, and a transcription terminator, all imbedded in the same construct.23,24 This means that the construct would express multiple sgRNA transcripts able to target multiple genomic loci. In the second approach, multiple sgRNAs are released from one single transcript, produced from either one RNA polymerase III promoter25 or one RNA polymerase II promoter.26 Such a polycistronic transcript offers the possibility of encoding additional exogenous factors to sgRNAs in the case of RNA polymerase II-driven promoters, although efficient processing of the transcript for releasing active sgRNAs and polyadenylated transcripts for nuclear export is rather complex in nature. First, sgRNA must be released from the primary transcript and, therefore, requires the addition of flanking sequences that are recognized by exogenous factors that must be provided in trans, such as Cys4 protein from Pseudomonas aeruginosa.26 Second, the remaining transcript must...
be properly processed as a translational active molecule. Additionally, a shortcoming of this system is that Csy4 has cytotoxic effects at high concentrations, a fact that might hinder the clinical scenario. Moreover, this number of supplemental sequences significantly hampers the efficiency of the targeting and editing capabilities of the CRISPR/Cas9 system because of the addition of extra variables.

Exploiting the endogenous RNA processing factors would be preferred, and simplistic approaches for multiplexing CRISPR/Cas9 have already been reported. In one study, the endogenous tRNA processing machinery was used for processing an sgRNA-tRNA polycistronic transcript to release multiple active sgRNAs. This approach is a powerful and reliable system for multiplexing CRISPR/Cas9 because it takes advantage of the abundance of endogenous cellular RNAses without heterologous expression of additional and potential cytotoxic factors that might interfere with normal cell function. This interference with normal cell function could also make the evaluation of results rather difficult and unreliable.

Having a system that can easily and efficiently target gene networks, multiplexing CRISPR/Cas9 offers the advantage of deleting genes or even inserting new genes in the genomes of choice. This provides a toolbox that opens new horizons that researchers could only dream of, with an ease never previously attained. The feasibility of CRISPR/Cas9 systems for knockin in reporter genes in different genomic sites has already been reported for different cell types, such as mouse haploid embryonic stem cells and human embryonic pluripotent stem cells. The authors used an HDR approach in which the reporter cassette was flanked by two homologous arms on the region of interest in the genome, and two sgRNAs were used for guiding Cas9 nuclease to the targeted sites. Upon cleavage, the reporter cassette is inserted in the genome by homologous recombination with an efficiency that varies depending on the targeted site. Using this approach, studies have shown that such a procedure permits the generation of mice harboring reporter genes up to 5 kb or a conditional allele with ease, surpassing traditional approaches in terms of simplicity, time length, and cost-effectiveness. The only requirement is the presence in the donor plasmid of two flanking homologous arms that may vary in length from 500 bp to 900 bp and two sgRNAs sequences able to direct Cas9 toward the targeted genomic fragment. In the absence of a donor DNA, large genomic deletions up to 65 kb can be easily generated, making CRISPR/Cas9 a preferred system for obtaining knockout mouse models for translational research.

CRISPR/Cas9 Libraries

Taking advantage of the CRISPR/Cas9 system’s ability to target almost any genetic loci within a target cell, researchers have created entire libraries of sgRNAs that are directed toward specific genes to examine the meaning of a “custom-made” phenotype in different experimental setups. By introducing into cells sgRNA libraries via delivery vectors that are most often lentiviral particles, there is the possibility to repress, activate, or even knock out different target genes at once to elucidate their function within a pathologic or homeostatic context. The extensiveness of the library is also a critical factor, where there is the possibility to target the whole genome with the use of a single, large “pooled” library of sgRNAs or to target specific subsets of genes that are thought to be interconnected, known as sub-pooled libraries. Moreover, the permanent nature of the genetic modifications mediated by CRISPR/Cas9 technology permits the evaluation of the modified cells for extended periods of time, where there is the possibility that a certain phenotype will manifest only after several cell divisions.

The first step when performing a screening investigation is viral administration of the selected sgRNAs in cells that consistently expresses Cas9 enzyme or in combination with the Cas9-encoded sequence under the construct. After selection of the transfected cells, further selective agents can be added that will enrich the resistant phenotype population. The final step consists of sequencing arrays that can reveal the difference between the experimental and control groups in terms of enriched sgRNAs and depleted ones, which can be seen in Figure 3. When these data are overlapped with the original sgRNA library and screened for the target genes, statistical analysis can reveal the key genes within the phenotype of concern.

Shalem et al. and Wang et al. pioneered the genome-wide screening of cells using CRISPR/Cas9 libraries and presented the advantages of this method in contrast to small interfering RNA. Although their approaches were quite different, where Shalem et al. transfected cells with a vector containing both sgRNAs and a Cas9-expressing sequence, and Wang et al. administered viral particles containing only sgRNAs molecules in cells that already stably expressed Cas9 enzyme, the results proved encouraging for both groups. Thus, even though this approach for the identification of entire genes set functions in living cells is still in its infancy, CRISPR/Cas9 libraries hold significant advantages over the preceding technologies (e.g., small interfering RNA [siRNA libraries]), allowing the generation of a permanent “mutated” phenotype that can be studied across several cell division steps under different experimental conditions.

The Big Step of CRISPR/Cas9 in Cancer Research

Cancer is one of the most investigated fields in medicine because of its high prevalence in the human population, the complexity and heterogeneity of the disease, which makes current treatments rather unspecific, raising the death toll of the affected individuals. Understanding the molecular mechanisms that underlie the proliferative process is a prerequisite for the development of reliable experimental models in which the altered cellular pathways can be reproduced and novel targeted therapies can be implemented. Hence, it did not take long until the genome editing capabilities of CRISPR/Cas9 proved its utility in cancer research and development of therapeutical strategies. Such applicability ranges from functional validation of genes implicated in cancer development and progression to cancer modeling and therapeutic concepts, as described in Table 3. Therefore, CRISPR/Cas9 has spread to every aspect of cancer research in
a very short period of time, making it a versatile technology that could leave an important footprint with regard to redefining the meaning of cancer biology and its treatment. From small-scale studies to high-throughput screens of gene function, CRISPR/Cas9 has brought new insights into cancer progression and metastasis and led to the identification of new potential therapeutic targets. Cancer modeling has an opportunity with CRISPR/Cas9 for both in vitro and in vivo models because the traditional Cre/LoxP recombination technology for animal models has shortcomings in terms of labor, time length, and costs for producing stable in vivo lines of cancer models. In addition, this might bypass tumor xenograft models, where the risk of immune incompatibility can interfere with the production of reliable in vivo models. The use of immunocompromised animals requires special conditions for maintenance of the lines, and lack of an immune response might interfere with the results obtained in preclinical studies, a crucial step for evaluating the safety profile of novel therapeutic agents.

A few studies published recently on in vitro and in vivo cancer models have paved the way for CRISPR/Cas9 in therapeutic applications for the oncology niche. In this regard, cancer immunotherapy gained special attention by reprogramming T cells through disruption of the programmed death-1 receptor (PD-1) over conventional PD-1 antibody therapy. Because the PD-1 ligand is a negative regulator of T cell activity and expressed on dendritic cells as well as in some tumors, knocking down PD-1 with CRISPR/Cas9 could reprogram T cell activity toward PD-1 ligand-expressing tumors. This approach is already in phase I clinical trials for castration-resistant
| Cell Line/Organism | Delivery System | Cancer Type | Targeted Gene | Local/Systemic | Reference |
|--------------------|-----------------|-------------|---------------|----------------|-----------|
| **Gene Function Studies In Vitro** | | | | | |
| A375 melanoma cell line (human) | LV | melanoma | SAM library | | 65 |
| A375 melanoma cell line (human) | LV | melanoma | GeCKO library | | 45 |
| Non-small-cell lung cancer cell line | LV | non-small-cell lung cancer | GeCKO library | | 64 |
| MDA-MB-231, MCF-7 (human) | LV | triple-negative breast cancer | Shcp1 | | 65 |
| T24, J82, 5637, SW-780 bladder cancer cell lines (human) | plasmid transfection | bladder cancer | p21, E-cadherin, hBax | | 65 |
| HEK293T (human) | plasmid transfection | non-small-cell lung cancer | Cd74-Ros1 | | 64 |
| HEK293 (human) | plasmid transfection | non-small-cell lung cancer | Eml4-Alk, Ki55-Ret | | 65 |
| Dld-1 (human) | plasmid transfection | colon cancer | Pkc | | 65 |
| B16-F10, Ret melanoma cell lines (mouse) | plasmid transfection | melanoma | Id1, Id3 | | 67 |
| BT-474, SKBR-3, MCF-7 (human) | RV/plasmid transfection | breast cancer | Her2 | | 68 |
| **In Vivo** | | | | | |
| Mouse | LV | prostate cancer | TGFBRII | ex vivo | DU145 cells (human) | 66 |
| Mouse | LV | prostate cancer | Nanog1 | ex vivo | DU145 cells (human) | 66 |
| Mouse | LV | triple-negative breast cancer | Cripto-1 | ex vivo | MygMC(A) (mouse) | 67 |
| Mouse | LV | triple-negative breast cancer | Cbfa1 | ex vivo | MDA-Mb-231 (human) | 68 |
| Mouse | plasmid transfection | cervical cancer | HPV E6, E7 | ex vivo | SHa, C33-A (human) | 69 |
| Mouse | LV | Burkitt lymphoma | Mcl-1, TP53 | ex vivo | HSC (mouse) | 70 |
| Mouse | plasmid transduction | acute myeloid leukemia | Mll3 | ex vivo | HSC (mouse) | 70 |
| **Cancer Modeling In Vitro** | | | | | |
| Myoblast cells (mouse) | LV | alveolar rhabdomyosarcoma | Pax3, Foxo1 | | 75 |
| HEK293A, hMSCs, PBMCs, HL-60 (human) | plasmid transduction/EP | Ewing sarcoma, acute myeloid leukemia | Ewsr1, Flk1, Runx1, Eto | | 75 |
| **In Vivo** | | | | | |
| Mouse | LV | non-small-cell lung cancer | Emi4-Alk | local | 75 |
| Mouse | LV | non-small-cell lung cancer | Nks2.1, Pten, Apc | local | 75 |
| Mouse | LV/Ad | pancreatic ductal adenocarcinoma | Lkb1 | local | 75 |
| Mouse | Ad | non-small-cell lung cancer | Emi4-Alk | local | 75 |
| Mouse | AAV | lung adenocarcinoma | TP53, Lkb1, Kras | local | 75 |
| Mouse | HI | hepatocellular carcinoma | multiple | systemic | 81 |
| Mouse | HI | intrahepatic cholangiocarcinoma | | | 81 |
| Mouse | HI | hepatocellular carcinoma | Pten, TP53 | systemic | 81 |
| Mouse | EP | glioblastoma | Pten, Apc, Nf1 | local | 82 |

(Continued on next page)
has and will continue to benefit from this new genome editing technology. In particular, modeling oncogenesis in mice using CRISPR/Cas9 nuclease系统 opened the door to new possibilities in cancer research.43 The simplicity that resides in the genome editing capabilities of CRISPR/Cas9 attracted the attention of many researchers around the world, as confirmed by the overwhelming numbers of studies that have been published on the subject for the past 2 years. This newly characterized RNA-guided Cas9 nuclease system opened the era of “molecular surgery,” and, as reviewed above, cancer research has and will continue to benefit from this new genome editing technology. In particular, modeling oncogenesis in mice using CRISPR/Cas9 surpassed previous genome editing tools in terms of time length and cost effectiveness without the need of multiplying colonies of mice.

Functional studies of multiple genes in cancer cell lines with CRISPR/Cas9 (Table 3), especially large-scale studies with sgRNAs libraries (Table 3),43-45 again stressed the complexity of this insidious killer that makes cancer particularly difficult to treat. The multigenic and multi-mutated status gives cancer an unique heterogeneity characteristic; this heterogeneity is even seen in different individuals affected by the same cancer type,51,52 and this truly represents the major challenge for clinical therapy. Although surgery and chemotherapy/radiation therapy are currently in clinical use for the treatment and palliative care of cancer patients, the lifespan, because of tumor relapse, is quite short, and the quality of life because of adverse effects of therapy is rather poor. All of this gave researchers the impulse to find alternative treatments that are efficient for targeting the diseased cells within our body, and gene therapy emerged as a vision for treatment of human diseases that would be safe and specific.

Most of the biotherapies are based on oncolytic vectors that selectively replicate and destroy cancer cells, releasing tumor-specific antigens, resulting in a second immune-humoral response against distant metastatic tumors.53 However, these therapies are limited to local administration, a fact that imposes several limitations, such as tumor localization, that require invasive approaches that are not feasible in several cancer types. For the time being, CRISPR/Cas9 has been investigated as a potential therapeutic strategy in ex vivo preclinical setups for cancer immunotherapy in both hematological and solid tumors by targeting the PD-1 gene in T cells (Table 3), reprogramming these cells of the immune system to recognize and attack cancer cells. To date, there are four clinical trials under investigation based on the strategy of targeting PD-1 by CRISPR/Cas9 in four different cancer types (https://clinicaltrials.gov). Both the preclinical and clinical studies are using lentiviruses (LVs) as delivery systems in T cells of the CRISPR/Cas9 components. For ex vivo therapeutic setups, transduction of primary T cells from cancer patients, characterization, and expansion in the laboratory of the transduced cells are quite labor-intensive and expensive. Although lentiviruses have been reported to be efficient for tumor regression on in vivo models, with CRISPR/Cas9 or other experimental setups, like utilizing antagonists,54 a valuable lesson has been learned in terms of the tumorigenic potential of integrating vectors in clinical trials.55

Having a technology that gives us the possibility to knock out or knock in single or multiple genes with an ease that surpasses other genome editing tools is clearly a major achievement. Furthermore, researchers have engineered Cas9 nuclease into an RNA-guided
transcriptional activator by fusing the VP64 transcription activation domain to a catalytically inactive Cas9 and linking a MS2-p65-HSF1 activation complex to sgRNA via a hairpin aptamer. Rendering sgRNA from 20 bp to 11–15 bp, an RNA-guided transcriptional activation complex, Cas9-MS2-p65-HSF1, was obtained with an active Cas9. This system offers the possibility of simultaneous orthogonal activation of gene expression with gene knockout by "classical" Cas9-sgRNA system in a single cell population. The specificity of such an approach can be further enhanced by substituting the Streptococcus pyogenes Cas9 (spCas9) with the Streptococcus aureus Cas9 (saCas9); this allows for recognition of the PAM of the consensus sequence NNGRR(T), which is more complex than the NGG spCas9 PAM. SaCas9 also has a significantly smaller size than spCas9, a particularity that might be useful when physical constraints are imposed by the type of vector that is used as the delivery vehicle. However, such a complex PAM reduces the number of genomic loci that can be targeted.

Despite the numerous efforts that have been made to improve the specificity and efficiency of CRISPR/Cas9, a major hurdle experienced with other gene therapy approaches lies before us. Delivery into the body and targeting the diseased cells without side effects is not something new, and each type of delivery vector has its own advantages and limitations. Therefore, a safe and efficient delivery vector for systemic administration of CRISPR/Cas9 would be highly desirable. An emerging class of gene delivery vectors is derived from an M13 filamentous bacteriophage in which a tumor-targeting ligand is expressed on the bacteriophage capsid. Because bacteriophages do not have natural receptors on human cells, attaching a ligand like Arg-Gly-Asp (RGD) confers an improved specificity for tumors over conventional vectors derived from human viruses, which need complex engineering of the capsid to limit the tropism for their cognate receptors. In addition, bacteriophage-derived vectors could bypass the immune response, commonly experienced with adenoviral vectors, in which a pre-existing immunity against adenoviral strains can result in severe life-threatening allergic side effects, as previously reported in the classic case of the Jesse Gelsinger gene therapy trial.

Furthermore, M13 bacteriophage-derived vectors can accommodate into their genome exogenous genetic material up to 13 kb without affecting their 3D structure, an important aspect to consider when designing an all-in-one vector; the physical constraint experienced with AAV delivery vectors limits the cloning capacity to 4 kb. Differential microRNA expression patterns between the tumor and normal tissue can be exploited to restrict Cas9 activity to cancer cells where a specific miRNA is downregulated by the inclusion of miRNA targeting sequences in the 3’ UTR of Cas9, further increasing the specificity of the bacteriophage-derived vector for cancer cells. Likewise, using a tumor-specific promoter to drive Cas9 expression can lead to an increase of CRISPR/Cas9 specificity for cancer cells.

Is the bacteriophage-derived vector the vehicle that will move CRISPR/Cas9 to clinical applications in a simple and efficient manner without the potential genotoxic effects of integrating vectors? Perhaps, but we cannot envision such a scenario at this time because more preclinical studies must be implemented to test its clinical significance and evaluate the pharmacokinetic properties of phage-derived nanoparticles. However, we must keep in mind that the multigenic and heterogeneous nature of cancer is definitively the major challenge for an efficient therapy. Even with a systemic delivery vector that can efficiently deliver CRISPR/Cas9 to cancer cells without side effects, there is no guarantee that a full therapeutic effect will be achieved. A thoughtful understanding of the proliferative process and connections between different cellular pathways in cancer cells is mandatory for developing efficient therapeutics, especially considering that each type of cancer has its own genomic and phenotypic profiles. Whether CRISPR/Cas9 technology will make a difference in cancer therapeutics is hard to tell, but it is definitely worth the effort. And the knowledge that will arise from this process of “trial and error” will pave the way for CRISPR/Cas9 in our daily medical practice and, if not for cancer, then perhaps for other diseases that are threatening our own existence and well-being.

CONFLICTS OF INTEREST
The authors declare no conflict of interest.

ACKNOWLEDGMENTS
We would like to thank Mihail Buse for English proofreading of the manuscript. This work was supported by national grants Nr.128,014-PN-II-PT-PCCA-2013-4-2166 (GenCanD) and POC Nr.35/01.09.2016, ID 37_796 (CANTEMIR).

REFERENCES
1. Gupta, R.M., and Musunuru, K. (2014). Expanding the genetic editing tool kit: ZFNs, TALENs, and CRISPR-Cas9. J. Clin. Invest. 124, 4154–4161.
2. Sander, J.D., and Joung, J.K. (2014). CRISPR-Cas systems for editing, regulating and targeting genomes. Nat. Biotechnol. 32, 347–355.
3. Khan, F.A., Pandupuspitasari, N.S., Chun-Jie, H., Ao, Z., Jamal, M., Zohaib, A., Khan, F.A., Hakim, M.R., and Shulgun, Z. (2016). CRISPR/Cas9 therapeutics: a cure for cancer and other genetic diseases. OncoTarget 7, 52541–52552.
4. Hsu, P.D., Lander, E.S., and Zhang, F. (2014). Development and applications of CRISPR-Cas9 for genome engineering. Cell 157, 1262–1278.
5. Sontheimer, E.J., and Barrangou, R. (2015). The Bacterial Origins of the CRISPR Genome-Editing Revolution. Hum. Gene Ther. 26, 413–424.
6. Sanchez-Rivera, F.J., and Jacks, T. (2015). Applications of the CRISPR-Cas9 system in cancer biology. Nat. Rev. Cancer 15, 387–395.
7. Fu, Y., Foden, J.A., Khayter, C., Maeder, M.L., Reyon, D., Joung, J.K., and Sander, J.D. (2013). High-frequency off-target mutagenesis induced by CRISPR-Cas9 nucleases in human cells. Nat. Biotechnol. 31, 822–826.
8. Hsu, P.D., Scott, D.A., Weinstein, J.A., Ran, F.A., Konermann, S., Agarwala, V., Li, Y., Fine, E.J., Wu, X., Shalem, O., et al. (2013). DNA targeting specificity of RNA-guided Cas9 nucleases. Nat. Biotechnol. 31, 827–832.
9. Xu, H., Xiao, T., Chen, C.H., Li, W., Meyer, C.A., Wu, Q., Wu, D., Cong, L., Zhang, F., Liu, J.S., et al. (2015). Sequence determinants of improved CRISPR sgRNA design. Genome Res. 25, 1147–1157.
10. Doench, J.G., Fusi, N., Sullender, M., Hegde, M., Vainberg, E.W., Donovan, K.F., Smith, I., Tothova, Z., Wilen, C., Orchard, R., et al. (2016). Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. Nat. Biotechnol. 34, 184–191.
22. Kleinstiver, B.P., Pattanayak, V., Prew, M.S., Tsai, S.Q., Nguyen, N.T., Zheng, Z., and Chen, Y., Liu, X., Zhang, Y., Wang, H., Ying, H., Liu, M., et al. (2016). A Self-restricted

21. Truong, D.J., Kühner, K., Kühn, R., Werfel, S., Engelhardt, S., Wurst, W., and Ortiz, Sakuma, T., Nishikawa, A., Kume, S., Chayama, K., and Yamamoto, T. (2014).

18. Liu, K.I., Ramli, M.N., Woo, C.W., Wang, Y., Zhao, T., Zhang, X., Yim, G.R., Chong, Ji, Y., Jin, H., et al. (2015). Large genomic fragment deletions and insertions in mouse

16. Liu, K.I., Ramli, M.N., Woo, C.W., Wang, Y., Zhao, T., Zhang, X., Yim, G.R., Chong, Ji, Y., Jin, H., et al. (2015). Large genomic fragment deletions and insertions in mouse

13. Cao, J., Wu, L., Zhang, S.M., Lu, M., Cheung, W.K., Cai, W., Gale, M., Xu, Q., and Xu, Q., and Feng, D.X. (2016). SHCBP1 is over-expressed in breast cancer and is important in

11. Fu, Y., Sander, J.D., Reyon, D., Cascio, V.M., and Joung, J.K. (2014). Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. Nat. Biotechnol. 32, 279–284.

12. Dow, L.E., Fisher, J., O’Rourke, K.P., Muley, A., Kastenhuber, E.R., Livshits, G., Tschaharganeh, D.F., Socci, N.D., and Lowe, S.W. (2015). Inducible in vivo genome editing with CRISPR-Cas9. Nat. Biotechnol. 33, 390–394.

13. Cao, J., Wu, L., Zhang, S.M., Lu, M., Cheung, W.K., Cai, W., Gale, M., Xu, Q., and Yan, Q. (2016). An easy and efficient inducible CRISPR/Cas9 platform with improved specificity for multiple gene targeting. Nucleic Acids Res. 44, e149.

14. de Solis, C.A., Ho, A., Holehonnur, R., and Ploski, J.E. (2016). The Development of a Viral Mediated CRISPR/Cas9 System with Doxycycline Dependent gRNA Expression for Inducible In vitro and In vivo Genome Editing. Front. Mol. Neurosci. 9, 70.

15. Ghosh, A., Yue, X., Lai, Y., and Duan, D. (2008). A hybrid vector system expands adenovirus-associated viral vector packaging capacity in a transgene-independent manner. Mol. Ther. 16, 124–130.

16. Liu, K.I., Ramli, M.N., Woo, C.W., Wang, Y., Zhao, T., Zhang, X., Yim, G.R., Chong, B.Y., Gowher, A., Chua, M.Z., et al. (2016). A chemical-inducible CRISPR-Cas9 system for rapid control of endogenous gene editing. Nat. Chem. Biol. 12, 980–987.

17. Davis, K.M., Pattanayak, V., Thompson, D.B., Zuris, J.A., and Liu, D.R. (2015). Small molecule-triggered Cas9 protein with improved genome-editing specificity. Nat. Chem. Biol. 11, 316–318.

18. Truong, D.I., Kühner, K., Kühn, R., Werfel, S., Engelhardt, S., Wurst, W., and Ortiz, O. (2015). Development of an intermEDIATE split-Cas9 system for gene therapy. Nucleic Acids Res. 43, 6450–6458.

19. Polstein, L.R., and Gersbach, C.A. (2015). A light-inducible CRISPR-Cas9 system for control of endogenous gene activation. Nat. Chem. Biol. 11, 198–200.

20. Nihongaki, Y., Kawano, F., Nakajima, T., and Sato, M. (2015). Photoactivatable CRISPR-Cas9 for optogenetic genome editing. Nat. Biotechnol. 33, 755–760.

21. Chen, Y., Liu, X., Zhang, Y., Wang, H., Ying, H., Liu, M., et al. (2016). A Self restricted CRISPR System to Reduce Off-target Effects. Mol. Ther. 24, 1508–1510.

22. Kleinsteiver, B.P., Pattanayak, V., Prew, M.S., Tsai, S.Q., Nguyen, N.T., Zheng, Z., and Joung, J.K. (2016). High-fidelity CRISPR-Cas9 nuclease with no detectable genome-wide off-target effects. Nature 529, 490–495.

23. Kabadi, A.M., Ousterout, D.G., Hilton, I.B., and Gersbach, C.A. (2014). Multiplex CRISPR/Cas9-based genome engineering from a single lentiviral vector. Nucleic Acids Res. 42, e147.

24. Sakuma, T., Nishikawa, A., Kume, S., Chayama, K., and Yamamoto, T. (2014). Multiplex genome engineering in human cells using all-in-one CRISPR/Cas9 vector system. Sci. Rep. 4, 5400.

25. Xie, K., Minkenberg, B., and Yang, Y. (2015). Boosting CRISPR/Cas9 multiplex editing capability with the endogenous RNA-processing system. Proc. Natl. Acad. Sci. USA 112, 3570–3575.

26. Nissim, L., Perli, S.D., Fridkin, A., Perez-Pinera, P., and Lu, T.K. (2014). Multiplexed and programmable regulation of gene networks with an integrated RNA and CRISPR/Cas toolkit in human cells. Mol. Cell 54, 698–710.

27. Kimura, Y., Oda, M., Nakatani, T., Sekita, Y., Monfort, A., Wutz, A., Mochizuki, H., and Nakano, T. (2015). CRISPR/Cas9-mediated reporter knock-in in mouse haploid embryonic stem cells. Sci. Rep. 5, 10710.

28. Merkle, F.T., Neuhausser, W.M., Santos, D., Yalen, E., Gagnon, J.A., Maas, K., Sandoe, I., Schier, A.F., and Eggan, K. (2015). Efficient CRISPR-Cas9-mediated generation of knockin human pluripotent stem cells lacking undesired mutations at the targeted locus. Cell Rep. 11, 875–883.

29. Zhang, L., Jia, R., Palange, N.J., Satheka, A.C., Togo, J., An, Y., Humphrey, M., Ban, L., Ji, Y., Jin, H., et al. (2015). Large genomic fragment deletions and insertions in mouse using CRISPR/Cas9. PLoS ONE 10, e0120396.

30. Yang, H., Wang, H., Shivala, C.S., Cheng, A.W., Shi, L., and Jaenisch, R. (2013). One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas9-mediated genome engineering. Cell 154, 1370–1379.

31. Agrotis, A., and Ketteler, R. (2015). A new age in functional genomics using CRISPR/Cas9 in arrayed library screening. Front. Genet. 6, 300.
axis suppresses drug resistance by simultaneous inhibition of cell survival and epithelial-mesenchymal transition in breast cancer. Oncotarget 7, 49859–49877.

71. Zhen, S., Hua, L., Takahashi, Y., Narita, S., Liu, Y.H., and Li, Y. (2014). In vitro and in vivo growth suppression of human papillomavirus 16-positive cervical cancer cells by CRISPR/Cas9. Biochem. Biophys. Res. Commun. 450, 1422–1426.

72. Aubrey, B.J., Kelly, G.L., Kueh, A.J., Brennan, M.S., O’Connor, L., Milla, L., Wilcox, S., Tai, I., Strasser, A., and Herold, M.J. (2015). An inducible lentiviral guide RNA platform enables the identification of tumor-essential genes and tumor-promoting mutations in vivo. Cell Rep. 10, 1422–1432.

73. Chen, C., Liu, Y., Rappaport, A.R., Kitzing, T., Schultz, N., Zhao, Z., Shroff, A.S., Dickens, R.A., Vakoc, C.R., Bradner, J.E., et al. (2014). ML3 is a haploinsufficient 7q tumor suppressor in acute myeloid leukemia. Cancer Cell 25, 652–665.

74. Lagutina, I.V., Valentine, V., Pichionne, F., Harwood, F., Valentine, M.B., Villarejo-Balcells, B., Carvajal, J.J., and Grosveld, G.C. (2015). Modeling of the human alveolar rhabdomyosarcoma Pax3-Fkhr chromosome translocation in mouse myoblasts using CRISPR/Cas9 nuclease. PLoS Genet. 11, e1004951.

75. Torres, R., Martin, M.C., Garcia, A., Cigudosa, J.C., Ramirez, J.C., and Rodriguez-Perales, S. (2014). Engineering human tumor-associated chromosomal translocations with the RNA-guided CRISPR-Cas9 system. Nat. Commun. 5, 3864.

76. Blasco, R.B., Karaca, E., Ambrogio, C., Cheong, T.C., Karayol, E., Minero, V.G., Voena, C., and Chiarle, R. (2014). Simple and rapid in vivo generation of chromosomal rearrangements using CRISPR/Cas9 technology. Cell Rep. 9, 1219–1227.

77. Sánchez-Rivera, F.J., Papagianakkopoulos, T., Romero, R., Tammela, T., Bauer, M.R., Bhukar, A., Joshi, N.S., Subbaraj, L., Bronson, R.T., Yue, W., and Jackson, T. (2014). Rapid modeling of cooperating genetic events in cancer through somatic genome editing. Nature 516, 428–431.

78. Chiu, S.H., Winters, J.P., Wang, J., Naranzan, S., Dudgeon, C., Tamburini, F.B., Brady, J.J., Yang, D., Gruner, B.M., Chuang, C.H., et al. (2015). Pancreatic cancer modeling using retrograde viral vector delivery and in vivo CRISPR/Cas9-mediated somatic genome editing. Genes Dev. 29, 1576–1585.

79. Maddalo, D., Mancho, E., Concepcion, C.P., Benetti, C., Vidigal, J.A., Han, Y.C., Ogrodowski, P., Crippa, A., Rekhtman, N., de Stanchina, E., et al. (2014). In vivo engineering of oncogenic chromosomal rearrangements with the CRISPR/Cas9 system. Nature 516, 423–427.

80. Platt, R.J., Chen, S., Zhou, Y., Yim, M.J., Swiec, L., Kempter, H.R., Dahlman, J.E., Parnas, O., Eisenhaure, T.M., Jovanovic, M., et al. (2014). CRISPR-Cas9 knockin mice for genome editing and cancer modeling. Cell 159, 440–455.

81. Weber, J., Ollinger, R., Friedrich, M., Ehmer, U., Barenboim, M., Steiger, K., Heid, I., Mueller, S., Maresch, R., Englertier, T., et al. (2015). CRISPR/Cas9 somatic multiplex mutagenesis for high-throughput functional cancer genomics in mice. Proc. Natl. Acad. Sci. USA 112, 15804–15808.

82. Xue, W., Chen, S., Yin, H., Tammela, T., Papagianakkopoulos, T., Joshi, N.S., Cai, W., Yang, G., Bronson, R., Crowley, D.G., et al. (2014). CRISPR-mediated direct mutation of cancer genes in the mouse liver. Nature 514, 380–384.

83. Zuckermann, M., Hovestadt, V., Knobbe-Thomsen, C.B., Zapatka, M., Northcott, P.A., Schramm, K., Belic, J., Jones, D.T., Tschida, B., Morvaz, B., et al. (2015). Somatic CRISPR/Cas9-mediated tumour suppressor disruption enables versatile brain tumour modelling. Nat. Commun. 6, 7391.

84. Maresch, R., Mueller, S., Veldkamp, C., Ollinger, R., Friedrich, M., Heid, I., Steiger, K., Weber, J., Englertier, T., Barenboim, M., et al. (2016). Multiplexed pancreatic genome engineering and cancer induction by transfection-based CRISPR/Cas9 delivery in mice. Nat. Commun. 7, 10770.

85. Heckel, D., Kowalczyk, M.S., Yudivich, D., Belizaire, R., Puram, R.V., Dickins, R.A., Vakoc, C.R., Bradner, J.E., et al. (2014). E-CRISP: fast CRISPR target site identification. Nat. Methods 11, 122–123.
88. Prykhozhij, S.V., Rajan, V., Gaston, D., and Berman, J.N. (2015). CRISPR multitargeter: a web tool to find common and unique CRISPR single guide RNA targets in a set of similar sequences. PLoS ONE 10, e0119372.

89. Doench, J.G., Hartenian, E., Graham, D.B., Tothova, Z., Hegde, M., Smith, L., Sullender, M., Ebert, B.L., Xavier, R.J., and Root, D.E. (2014). Rational design of highly active sgRNAs for CRISPR-Cas9-mediated gene inactivation. Nat. Biotechnol. 32, 1262–1267.

90. Pliatsika, V., and Rigoutsos, I. (2015). "Off-Spotter": very fast and exhaustive enumeration of genomic lookalikes for designing CRISPR/Cas guide RNAs. Biol. Direct 10, 4.

91. Stemmer, M., Thumberger, T., Del Sol Keyer, M., Wittbrodt, J., and Mateo, J.L. (2015). CCTop: An Intuitive, Flexible and Reliable CRISPR/Cas9 Target Prediction Tool. PLoS ONE 10, e0124633.

92. Montague, T.G., Cruz, J.M., Gagnon, J.A., Church, G.M., and Valen, E. (2014). CHOPCHOP: a CRISPR/Cas9 and TALEN web tool for genome editing. Nucleic Acids Res. 42, W401–W407.

93. Labun, K., Montague, T.G., Gagnon, J.A., Thyme, S.B., and Valen, E. (2016). CHOPCHOP v2: a web tool for the next generation of CRISPR genome engineering. Nucleic Acids Res. 44, W272–W276.