BY ERIC B. KMIEC, PHD

CRISPR-Directed Gene Editing in a Community Cancer Center

At a recent conference at the Vatican, Pope Francis reminded us that "not everything technically possible or doable is thereby ethically acceptable." When it comes to human gene editing, this statement is both timely and appropriate. The extraordinary speed with which the genetic tool Clustered Regularly Interspersed Palindromic Repeats (CRISPR) has entered the scientific arena and, in fact, the public discourse is astounding. This year, genome editing will be a central theme of the largest most influential biotechnology meeting in the world, BIO 2019, taking place in Philadelphia. The scientific sessions will include discussions of scale-up, manufacturing reimbursement, and perhaps most importantly, how innovative therapies will become accessible to all patients who seek such treatment. Rarely has a technological advance induced such widespread discussion in both scientific literature and the popular press.

With its simple design and elegant mechanism of action, geneticists often say that CRISPR has democratized human gene editing, because research labs throughout the world can design and utilize this tool without extensive training. However, it is one thing to be able to do something and quite another to be able to carry it out with high technical skill to avoid unintended consequences. As such, policymakers, ethicists, scientists, and the public are engaged in productive conversations about the regulation of CRISPR-directed gene editing. Having these important stakeholders take part in those conversations is a clear testament to the power of this technology and bodes well for its use as a game changer in the era of personalized medicine.

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In this article, I will discuss the emergence of gene editing as an approach to human genetic engineering and gene therapy, especially in the field of oncology, and why we should care about its rapid and often breathtaking development. I will discuss some of the challenges that remain in this young field, a field that has

made a surprisingly quick transition from bench to bedside. I will also touch upon the work that the Gene Editing Institute of the Helen F. Graham Cancer Center & Research Institute at Christiana Care Health System is doing to further research, education, and engagement with gene editing as a tool in the fight against cancer.

Breakfast Cereal or Breakthrough Genetic Tool?

Part of the popularity surrounding CRISPR likely arises in part from its acronym, which could be mistaken for a new type of breakfast cereal or a refrigerator feature that helps keep lettuce fresh. In reality, CRISPR is a string of nucleic acid bases (RNA) that pair with a cellular enzyme known as Cas9 (or related enzymes such as Cas12a) to form the active gene editing complex, CRISPR/Cas9 (see Figure 1, page 33). This complex is found in almost all bacteria,[1,2] where it is part of an adaptive immunity pathway used by bacterial cells to fight off viral infections (see Figure 2, page 33). For example, when a bacteriophage (virus) infects the cell, molecular scissors are activated and essentially chop up the infecting viral DNA.[3] The resulting fragments of viral DNA are inserted into the bacterial chromosome. Upon reinfection, these inserted viral segments instruct the bacterial cell that the same infection is beginning. The activated CRISPR complex then more rapidly fragments and destroys the incoming viral DNA. Simply stated, the bacterial cell remembers the first infection and is primed to attack during the second. In some ways, one can think of it as a form of bacterial vaccination.

The transition from a bacterial cell immunity pathway to human gene editing has evolved over the past five to seven years, when several laboratories began to experiment with CRISPR/Cas9 to either disable or repair human genes.[4,5]As is often the case in life, it is easier to destroy something than to repair it. Though we certainly would like to utilize gene editing to repair mutant genes, such as those involved in the pathogenesis of sickle cell anemia or cystic fibrosis, that repair event must be precise and is therefore more challenging.[6,7] CRISPR/Cas9 functions normally in the bacterial cell to only fragment, not repair or replace, the target DNA site, so it is quite an uphill struggle to achieve precise repair on side or offside corollary mutagenesis. This effect refers to CRISPR activity at non-targeted sites leaving behind a genetic scar or unintended genetic footprint. Most scientists believe that the most efficient use of CRISPR/Cas9 in human cells is obviously the destruction of the function of a gene, in a process known as *genetic knockout*. Though other cleavage complexes exist that do similar things (such as zinc finger nucleases and transcription activator-like effector nucleases[8]), CRISPR is the only tool that exists naturally. It also happens to be easier to synthesize and is likely to be able to be produced in levels great enough to enable the critical translational step of scale-up, an important, but often forgotten, step for human clinical applications.

The World Before CRISPR

Before CRISPR, it was largely believed that creating site-specific cleavage in human chromosomes was impossible, and research and development toward that goal was often met with significant criticism.[9,10] Conceptually, single-agent gene repair—or *gene editing*, as it is called today—takes place in a two-phase reaction: pairing/alignment and cutting/repairing/resolution. The major barrier to further development of gene editing was the low frequency with which gene editing events took place. Targeting of chromosomal DNA had been successful in yeast and bacteria, likely because one could employ a stringent selection process to identify converted clones. These selection protocols are less effective in mammalian cells, and the choice of selection agents is limited.

Because the frequency of gene repair in eukaryotic cells was so low, a significant focus was placed on modifying the metabolic pathways of the target cell to make it more amenable to gene editing activity. It became apparent to clinicians in the field that double-stranded DNA breaks catalyzed by anticancer drugs or programmable nucleases such as CRISPR could prepare the cell for higher levels of gene editing by altering the speed at which DNA replication takes place. Retarding the progression of these important cellular metabolic pathways enables the enzymes and regulatory factors to be prompted and stay active for longer periods of time. These same factors have now been shown to influence the frequency of CRISPR-directed human gene editing, so the field of genetic engineering is focused almost exclusively on CRISPR as a therapeutic agent for human gene editing.

CRISPR and Drug Discovery

David Wollenberg points out that pharmaceutical companies normally develop drugs to *reach* a broad spectrum of patient population; however, that goal cannot be called personalized (known as the "reach").[11] Diversity of patients is a key challenge for any broad-spectrum drug; this is even observed with new immunotherapy agents. The expanding databases that continue to educate us about the complexity of the human genome have brought about the possibility that we may be able to develop personalized therapeutics that can treat individuals on a case-by-case basis (known as the "richness"). Exciting, yes; practical, maybe. CRISPR-directed gene editing is at the forefront of this latter strategy, though significant technical challenges exist, particularly with the associated higher costs. Debate now swirls around who will pay for gene-edited cell therapies and when they should be utilized. CRISPR has already been utilized in diagnostic testing, including the identification of the Zika virus,[12] and as a sophisticated and accurate diagnostic assay that can advise primary care physicians as to the best course of treatment for an individual patient with cancer.[13] The field of cancer diagnostics is likely to evolve faster than cancer therapeutics, and it is possible that soon most effective cancer diagnostics will involve a gene editing component.

(continued on page 34)

Figure 1. The CRISPR/Cas9 Complex

The DNA helix illustrated in blue is bound by a specific piece of RNA known as CRISPR (cr)RNA, which is paired with a separate piece of RNA (tracrRNA) that localizes on a specific site on the DNA. The seed sequence of the crRNA consists of approximately 20 bases that align in homologous register to the specific DNA sequence of the target site. At one end of the target DNA sequence is the site known as Protospacer Adjacent Motif, which helps position the Cas9 protein (grey-shaded region) to execute DNA cleavage.

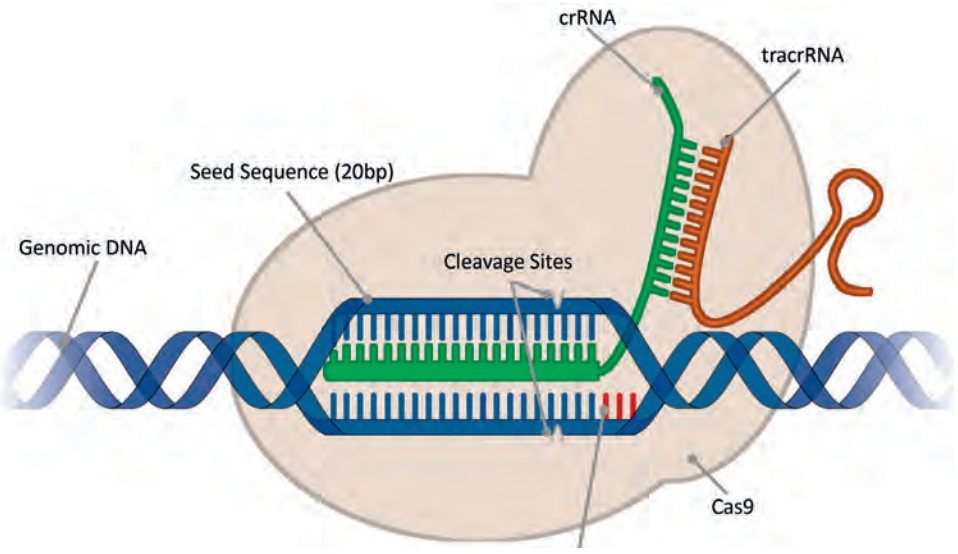
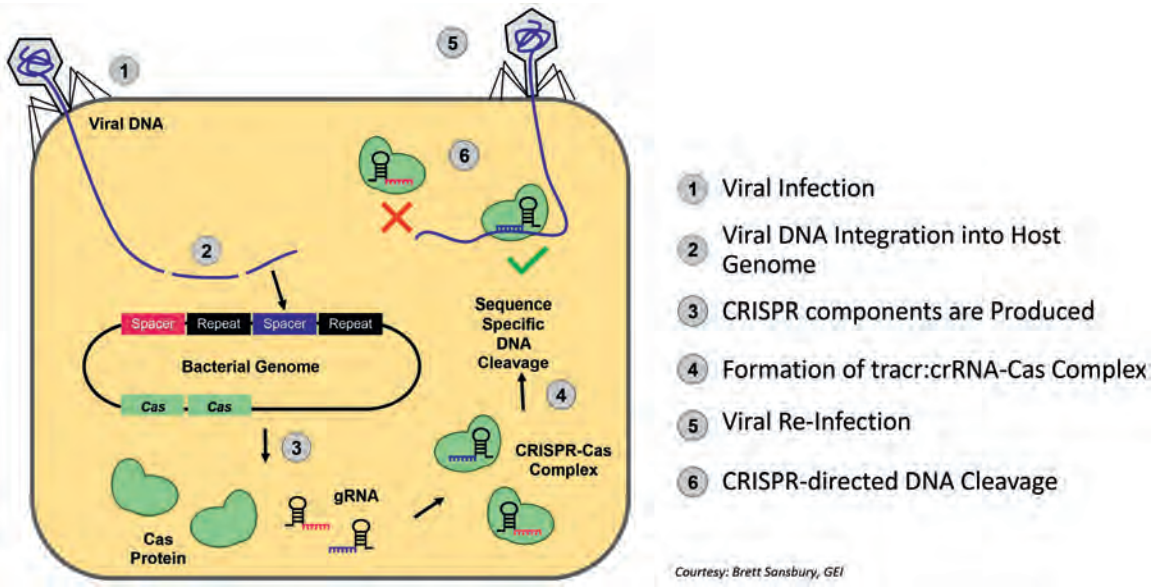


Figure 2. CRISPR/Cas9-Mediated Immunity in Bacteria

The infection and re-infection cycle is displayed with specific points where CRISPR is asked to fight off the viral infection. The explanation for each specific reaction step is placed on the right-hand side of the figure.



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CRISPR has already helped to re-identify the targets for certain well-known drugs. For example, a recent CRISPR/Cas9 screen for the essential genes involved in tumor growth led to the discovery that the MELK protein, known to be an essential for tumor growth, does *not* in fact drive cell proliferation in cancer cells as previously thought.[14] As the era of personalized medicine begins, it will be critical to validate potential (and now previously identified) drug targets by using screening methodologies that act at the level of gene by these more robust genetic techniques.

The Challenge of the Human Genome

CRISPR-directed gene editing has made huge inroads into the areas of cancer diagnostics, drug discovery, and cancer therapy, and it will have a direct impact on accelerating the development of personalized medicine for all forms of human disease. Yet, as with most rapidly accelerating technologies, fundamental challenges still exist, at both the basic and translational levels.

Though CRISPR is a highly precise genetic engineering tool, it does have the inherent capacity to bind and cleave at non-specific (off-target) sites in the human chromosome. It is likely that off-site mutagenesis will remain an open question, because ensuring a patient that no off-site mutagenesis will take place is simply impossible. We also know from the way that biological systems function that, by and large, nothing is perfectly precise, and go/no-go decisions, when considering whether CRISPR/Cas9 should be incorporated into a therapeutic regimen, may come down to a risk- and cost-benefit analysis. Alternatively, the disruption of the coding region of a mammalian gene is at the heart of the power of the CRISPR technology, and when the objective is to simply knock out the gene, as in most strategies for cancer therapy, the impact and even the importance of off-site or on-site mutagenesis are significantly reduced.

For the implementation of CRISPR-directed gene editing in human therapeutics, finding an appropriate target DNA sequence may not be the only molecular challenge. The genome is dynamic in that transcription, replication, repair, and DNA modification are taking place continuously throughout the chromosomes, and these reactions pose additional barriers to the accurate activity of CRISPR. When developing strategies for gene therapy, it will also be important to consider the epigenome, which generally refers to the degree of methylation within promoter regions and coding regions of human genes.[15] This work is in its embryonic stages and its true impact has not been established. However, most scientists agree that the inherent complexity of the human genome may pose additional barriers to success.

Lastly, with the excitement surrounding the evolution of CRISPR, it is often forgotten that this genetic tool only executes double-stranded breakage—the first step of gene editing. DNA resection, processing, and subsequent activities leading to gene knockout or gene knock-in are reliant upon the cell's endogenous DNA repair and replication pathways. Unfortunately, these pathways were not designed to facilitate the genetic re-engineering of human chromosomes, so when a double-strand break occurs, the cell assumes that a chromosome has been broken and needs immediate repair, which often takes the form of reconnecting the chromosomes no matter what the cost. This action often leads to a loss of DNA because the re-ligation process is notoriously unfaithful. Thus, once again, DNA deletion or gene disruption is a more attainable goal for CRISPR-directed gene editing. Another important response to DNA damage, often in the form of DNA breakage, is the activation of the tumor suppressor gene p53. There is no evidence that CRISPR-directed gene editing induces tumorigenesis, but because the DNA damage response includes the activation of these tumor suppressor genes, significant caution should be exercised when advancing novel therapies toward the clinic in the absence of a full analysis of gene expression.

The Gene Editing Institute at the Helen F. Graham Cancer Center & Research Institute

The Helen F. Graham Cancer Center & Research Institute at Christiana Care serves Delaware and neighboring communities. Christiana Care has one of the busiest cancer programs on the East Coast, treating more than 70 percent of the cancer cases in Delaware. More than 223,000 patient visits are recorded annually, and the Helen F. Graham Cancer Center & Research Institute is projected to treat more than 3,000 new cancer cases this year alone. Christiana Care has already become a national leader in cancer clinical trials, with 24 percent of patients enrolled in one or more clinical research trials for the prevention, early detection, and treatment of cancer, compared to the national average of 4 percent.

The Gene Editing Institute was founded at the Helen F. Graham Cancer Center & Research Institute in 2015 with four core missions:

1. Carry out grant-funded innovative translational research on the use of gene editing in cancer with a central focus on elucidating the transformation pathways as well as developing innovative technological approaches for studying oncogenesis.
2. Provide a focused educational resource for undergraduate and graduate students, postdoctoral fellows, and faculty interested in understanding and learning about gene editing.
3. Provide a biomedical resource facility for the synthesis, hands-on training, and dissemination of gene editing technologies to undergraduate institutions as well as to advanced research laboratories throughout the world.
4. Engage in sustainable partnerships with life science companies and research institutions through license deals and joint ventures.

The Gene Editing Institute can provide technical assistance for biomedical and agricultural researchers and other community cancer centers interested in utilizing gene editing technologies, and it is hoped that it will establish itself as a center for technology development and clinical implementation of gene editing, as well as an educational resource for developing curricula in gene editing.

The overarching strategy was to embed the Gene Editing Institute in a community cancer center, so that interactions with

physicians within a truly clinical environment could be facilitated. This structure has already afforded the opportunity for the development of meritorious translational research projects. Funding for the Gene Editing Institute comes from the National Institutes of Health, the National Science Foundation, the Binational Industrial Research and Development Foundation, and partnerships with a wide range of biotechnology companies. The diverse missions of the Gene Editing Institute position the institute as a foundational platform upon which expansion and partnerships with other hospitals and organizations can now take place.

Gene Editing and Non-Small Cell Lung Carcinoma

The application of CRISPR-directed gene editing for cancer therapy is only at its beginning, and many strategies will undoubtedly be changed as the amount of information surrounding clinical implementation accumulates. There are essentially two ways to approach the therapeutic challenge. First is to remove the cells from the body, re-engineer them using CRISPR, and introduce them into the body to specifically attack the tumor. Work at the University of Pennsylvania focuses on liquid tumors, wherein T-cells are genetically modified *ex vivo* and then reintroduced into the body for the treatment of acute lymphoblastic leukemia and chronic lymphocytic leukemia.[16] Often referred to as personalized cellular therapy, this therapeutic strategy has received the U.S. Food and Drug Administration's prestigious Breakthrough Therapy designation.

The second approach is more complicated, involving the development of a CRISPR-directed gene editing strategy for solid tumors. Since this approach will involve *in vivo* delivery to tumor tissue buried in the body, increased challenges that surpass liquid tumor applications are present. One such trial, however, is beginning this year and is focused on the action of CRISPR/Cas9 to target HPV 16 and HPV 18 E6/E7 DNA. The constructs will be delivered with a gel that is locally applied to the HPV infected cervix, which opens the possibility of deposition of CRISPR complexes following surgical resection. The focus at present is on safety, and dosing regimen and the change in HPV 16 or 18 will be evaluated in Phase 1. It should be noted that a very similar trial (NCT01800369) using zinc finger nucleases already has finished and is entering the data collection phase. So, some exciting developments are beginning in the treatment of solid tumors. It is widely recognized that over 85 percent of patients with lung cancer seek care at community cancer centers, and in our opinion, these patients should have access to innovative therapies at their treatment site. Though there is substantial progress in treatment modalities including immunotherapy, the treatment is far from ideal.[17] Several other approaches to using gene editing centered primarily on liquid tumors,[16] but the development of gene editing for solid tumors has lagged.

The Gene Editing Institute's combinatorial approach to treating KRAS+ non-small cell lung carcinoma, which includes CRISPR-directed gene editing, is novel, and it is hoped that it will enable new scientific discoveries as well as reveal new translational challenges early on in the process. A team of gene editing scientists and oncologists at the Helen F. Graham Cancer Center & Research Institute has been shaping realistic research goals that include an increase in chemosensitivity and an arrest of tumor growth, which will hopefully result in an improvement in survival rate and quality of life for patients with lung cancer. It is possible that this approach could be effective in patients with locally advanced lung cancer or could be incorporated in the early stage of disease, particularly with patients who have received surgical resection and/or are concurrently receiving immunotherapy or radiation therapy.

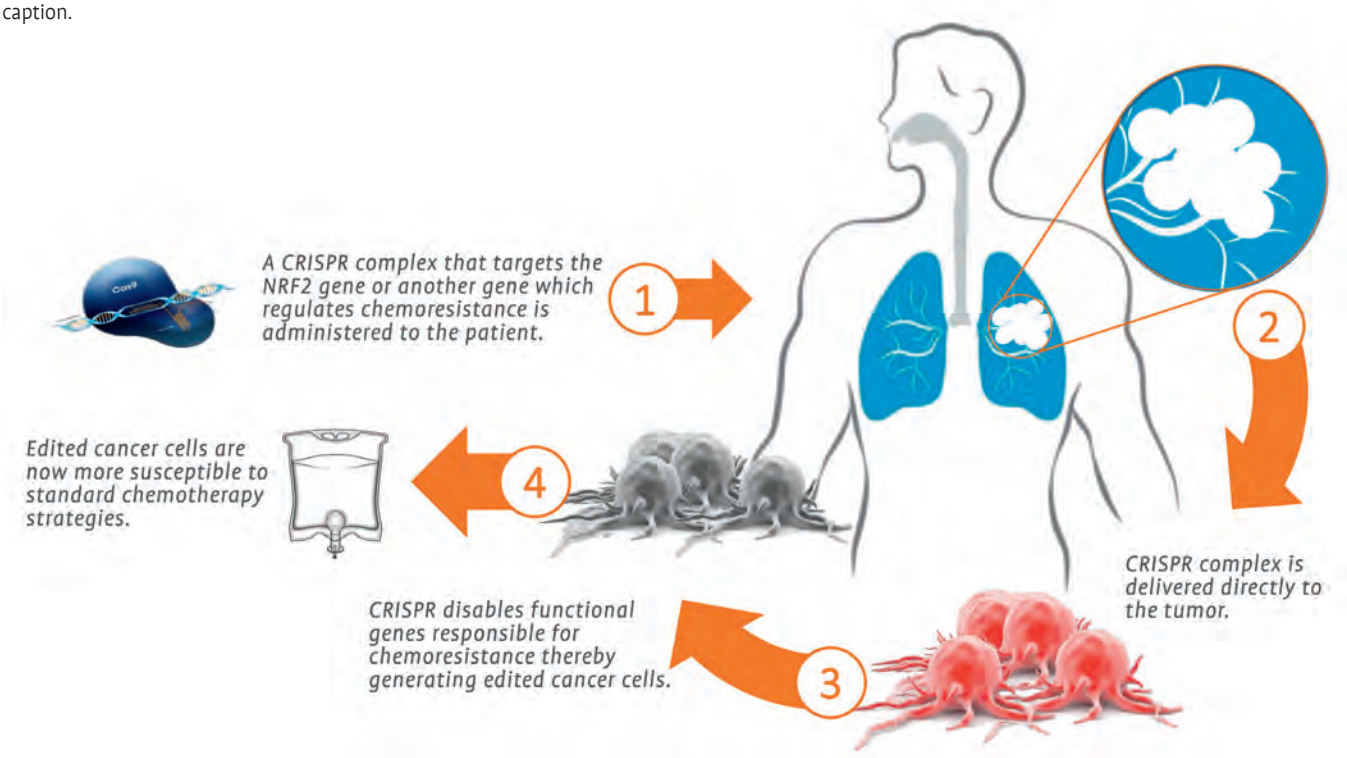
Chemotherapy remains an important option in the treatment of lung cancer, but issues involving chemoresistance and toxicity are often problematic with extended care.[18] Our goal is to establish a clear demonstration that genetic knockout of a gene encoding a transcription factor, such as NRF2 or any other gene controlling chemoresistance, improves the efficiency of chemotherapy. This discovery could potentially introduce a new weapon in the anticancer treatment armament. Because only 8 percent of NRF2 genes contain mutations in lung cancer, a set of established CRISPR molecules could be available on a standardized basis, turning this approach into an off-the-shelf therapy for most patients (see Figure 3, page 36).

Early results from this translational research project are quite encouraging; we can clearly observe an increase in chemosensitivity to cisplatin in a dose-dependent fashion in genetically re-engineered human lung cancer cells. Gene-edited human lung cancer cells proliferate at a slower rate than wild-type cells in the absence of drug treatment, but the combination of knockout cells and drug treatment leads to a cessation of tumor growth and maintenance of tumor size over the course of 16 days.

The major challenge of developing a CRISPR-directed gene editing for solid tumors such as lung cancer centers on one word: *delivery*. Despite billions of dollars of investment that have gone into the viral gene therapy arena, few viral vectors are suitable for tumor-specific delivery of therapeutic payloads especially to solid tumors. Several innovative strategies are being developed in order to provide a selective activity paradigm for CRISPR/Cas9 complexes designed to disable chemoresistance genes in lung tumor cells. There is mounting evidence that the DNA sequence of genomic targets within tumor cells is different than the DNA sequence of normal cells, and designing a CRISPR construct that specifically functions only when bound stably to the tumor cell DNA may sidestep the complexities and the well-known lack of specificity of viral or non-viral delivery systems. In addition, a rapidly emerging therapeutic delivery vehicle is the exosome, an extruded sub cellular particle surrounded by a membrane that can carry a variety of biomolecules. Tumor cells extrude these vesicles on a regular basis, and these particles returned to the tumor cell through some sort of molecular recognition. Thus, it is possible that exosomes can be captured from the primary tumor and packaged with the CRISPR/Cas9 complex, followed by targeted delivery to the tumor from whence the exosome arose.

Figure 3. A Potential Experimental Protocol for the Use of CRISPR-Directed Gene Editing for the Treatment of Non-Small Cell Carcinoma.

The CRISPR/Cas9 expression construct is introduced into the patient using a viral vector or by direct injection into the tumor. CRISPR-directed gene knockout takes place at specific target genes, such as NRF 2, to complement or sustain standard of care. Each step in the protocol is highlighted by the associated caption.



Can and Will Gene Editing be Regulated?

The regulatory landscape surrounding gene editing is both inconsistent and confusing. Very few regulations are in place, and no internationally agreed-upon rules have been laid down. In most cases, each country is evaluating how best to control the progression of gene editing in both somatic and ultimately germline activities on its own. The European Union has established a legal and regulatory framework for safeguarding the development of genetically modified organisms and protecting humans, animals, and the environment.[19] However, there is a fundamental question as to whether the CRISPR/Cas9 activity at the level of the chromosome is, in fact, true genetic alteration—in most cases, no additional DNA is added to the genome. The argument can be made that the CRISPR technique itself should not be regulated, but rather only the product.

CRISPR-directed gene editing has also accelerated the discussion surrounding the modification of germline cells such as eggs, sperm, fertilized eggs, and embryos due to its efficiency and precision. However, gene editing of human embryos faces significant and fundamental barriers. Germline editing is banned in Canada, and experiments involving germline editing in Germany are currently limited by the Embryo Protection Act, which prohibits using human embryos for basic research and the harvesting of embryonic cells. South Korea's Bioethics and Biosafety Act also prohibits genetic experimentation on human embryos. In 2017, the U.S. National Academy of Sciences and the National Academy of Medicine opened the door slightly by recognizing the potential for using gene editing in embryonic cells to treat serious genetic diseases in cases where embryo editing is the only reasonable option. There was also consensus support for carrying out basic research in embryo editing, but such experiments are prohibited using federal funds—there is a congressional prohibition on using taxpayer funds for research that destroys human embryos.[20] No clinical trial of human editing will be approved by the U.S. Food and Drug Administration.

The challenges of applying CRISPR-directed gene editing for human disease in a community cancer center are much higher when compared to those encountered at major medical centers that associated biomedical research arms. Resources are often limited, and embedded expertise is lacking. Yet, most patients seek treatment at community cancer centers and it raises the question of accessibility. Are the rapidly developing gene editing

therapies going to be available only to those people who can readily access them repeatedly? Such a situation creates another healthcare disparity and a bifurcation of treatment options. These therapies are going to be expensive and certainly risky. Thus, innovative therapeutic development should be carried out at community cancer centers so that the uniqueness of the population and its associated diversity can be incorporated into therapeutic design. There appears to be no reason why variance of Gene Editing Institute structure cannot be created and localized in community cancer centers to work together with oncologists who see the wide diversity of patients seeking cancer care. We hope that our model will begin a conversation as to how best to improve the accessibility of such breakthrough technologies to those who most need it.

In closing, it is informative and proper to return to the statement by Pope Francis: "Not everything that is technically possible or doable is ethically acceptable." CRISPR is a generational technology that can enable remarkable genetic engineering to treat, cure, and even prevent human disease, and first in line could be various forms of cancer. Most scientists draw a distinct line between somatic cell gene editing and germline cell gene editing, so treating cancer with some form of gene editing will remain the most approachable therapeutic strategy. However, as clinical applications of CRISPR mature and safety concerns wane, the question may turn itself around and become: Is it ethically acceptable *not* to do what is technically possible? OI

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