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Current prospects and outlook of CRISPR/Cas technology in clinical therapy

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Current prospects and outlook of CRISPR/Cas technology in clinical therapy

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Abstract:

Gene editing capabilities have expanded enormously since researchers first demonstrated the robust, accurate, and efficient endonuclease activity of the CRISPR/Cas riboprotein enzyme. Since this gene editing technique was first described in 2013, the prospect of gene therapy as a viable clinical tool has improved immensely. CRISPR/Cas techniques and the methods by which they are delivered into cells, have evolved rapidly since the technology's inception, and successful, intentional genetic alterations of numerous mammalian cell lines have been reported. As a research tool, CRISPR/Cas9 has already proven itself indispensable in a brief period of time. While there are not numerous clinical trials involving CRISPR/Cas technology, CRISPR has absolutely contributed to the acceleration and expansion of gene therapy-based clinical trials in the past five years. Here, the current capabilities of CRISPR/Cas gene editing are evaluated as they relate to the clinical setting, including potential for CRISPR/Cas to function as: a new cancer therapeutic agent, a means of correcting or relieving genetic disease, and a potent disrupter of antimicrobial resistance genes in virulent MDR bacterial strains. Additionally, the future possibilities of CRISPR/Cas-related therapies, and issues preventing the achievement of these therapies in the clinical setting, are discussed.

Introduction:

In late 2017, Brian Madeux, an American man with the inherited genetic disorder of Hunter's syndrome, received a novel treatment at the Benioff Children's Hospital at the University of California–San Francisco – in vivo genetic editing, via the delivery of zinc finger nucleases (ZFNs) – to treat his disease. This represented the first report in the world describing the treatment of genetic diseases through in vivo gene editing, demonstrating that gene editing has important clinical potential for the treatment of genetic diseases[1]. Since that time, a new gene editing technique called CRISPR/Cas has emerged as an invaluable research technology, having been utilized in numerous clinical trials for the treatment of diseases including, but not limited to: sickle cell disease, Beta-thalassemia, heritable cardiomyopathies, lymphomas, melanomas, and viral infections such as HIV and Hepatitis B.

Researchers have known for decades that the genomes of bacteria and archaea housed sequences of bacteriophage DNA in patterns of clustered, regularly-interspaced palindromic repeats (CRISPR). These CRISPR segments, along with the CRISPR-associated (Cas) family of endonuclease enzymes, were understood to be a secondary microbial defense mechanism against invading bacteriophage viruses. Specifically, CRISPR/Cas9 is a type II acquired immune system in bacteria and archaea, and it serves to fend off cellular invaders by destroying bacteriophage DNA/RNA that a given phage has injected into the host microbe[2,3]. After destroying an invading bacteriophage, a bacterium can incorporate into its own DNA a small portion of the phage's DNA, and the incorporated phage DNA is thereafter termed a "spacer". While DNA normally acts primarily as a template for DNA replication or protein synthesis, the phage DNA spacer is transcribed into RNA and embedded within a Cas protein where it functions as a template for DNA destruction. In short, the spacer within the bacterial genome enables the host bacteria to

quickly recognize and defend against bacteriophage infections. A helpful analogy would be to think of a given Cas protein as a police officer, the CRISPR locus on a bacterial chromosome as a series of mugshots, and cytosolic DNA or RNA as a criminal suspect; as the Cas enzyme (the officer) compares free-floating DNA or RNA (suspects) to the spacer-derived RNA (the mugshot), it can recognize and neutralize dangerous "suspects" before any damage is done.

Cas enzymes need a guide RNA to specify their target. Naturally occurring Cas enzymes within microbes require two RNA sequences: trans-activating CRISPR RNA (tracrRNA) which enables Cas endonuclease activity, and CRISPR RNA (crRNA) which is derived from the spacer and provides the template with which the Cas enzyme targets a specific bacteriophage DNA sequence[3]. Natural Cas proteins destroy invading DNA by inducing a double strand break (DSB) in the foreign DNA sequence specified by guide RNA, after guide RNA dimerization with the exogenous target DNA[4].

In the laboratory, the same principle applies but with a custom-made synthetic single guide RNA (sgRNA), which is essentially a fusion of the tracrRNA and crRNA (Figure 1) [5,6]. In most cells, eukaryotic or prokaryotic, most DSB events are followed by cell-mediated DNA repair, which occurs in either an error-free pathway of Homology Directed Repair (HDR) or an error-prone Non-Homologous End-Joining (NHEJ) pathway[7,8]. The HDR pathway typically uses a sister chromatid as a template to repair a damaged gene segment in eukaryotic cells, thereby restoring the gene and its function. Repair by NHEJ, however, commonly features loss of nucleotides on either side of the DSB during the repair process, so when ligation finally occurs, the restored chromosome has either lost or needlessly added some genetic information; this introduces a nonsense mutation into the gene about 67% of the time, effectively knocking-out the gene by irreversibly altering the coding sequence[8] (Figure 2). NHEJ repair of a DSB can occur at

any time, but error-free HDR is isolated to G2 and S phases of the cell cycle[9]. While there are exceptions, CRISPR/Cas techniques that integrate an exogenous gene of interest into a host genome – i.e. a genetic knock-in event – generally rely on the HDR pathway for integration, but rely on the error-prone NHEJ pathway when CRISPR/Cas is utilized to knock-out an endogenous gene.

The first reports of researchers successfully modifying mammalian cells with CRISPR-Cas9 technology were published in 2013 by Jennifer Doudna's lab at University of California, Berkeley[1]. Since then, CRISPR/Cas technology has become a linchpin method for genetic research in laboratories all over the world and has supplanted previously popular enzyme-based genetic modification procedures such as Transcription Activator-Like Effector Nucleases (TALEN) and the previously mentioned ZFNs[10,11].

One main reason CRISPR-Cas9 is currently in vogue among researchers is due to its flexibility and elegant mechanism of action. Proteins are responsible for the enzymatic induction of DNA double-strand breaks (DSBs) in the CRISPR/Cas, ZFN, and TALEN gene-editing systems alike. In the ZFN and TALEN systems, proteins are also responsible for recognizing the correct target DNA sequence; contrast that with the CRISPR/Cas, wherein RNA is responsible for recognizing the correct DNA sequence[11] (Figure 3). Because targeting a new DNA sequence depends on protein alterations in the ZFN-based and TALEN-based nuclease systems, they both require extensive re-engineering for every unique DNA sequence they target. Changing the constituent proteins in ZFN and TALEN systems is somewhat fickle, and though ZFN and TALEN are well-studied, each modification can have unintended consequences that reduce their enzymatic efficiency or accuracy. Consequently, the re-designing process for ZFN and TALEN systems is time consuming, costly, somewhat unpredictable, and labor intensive.

The Cas family of enzymes, however, do not require any modifications to induce a DSB at a given sequence. Instead, a sgRNA embedded within the Cas enzyme recognizes and binds a complimentary DNA sequence, and the resultant extrinsic-DNA/intrinsic-RNA heterodimer triggers Cas-mediated DSB induction in the target DNA strand by hydrolyzing the covalent bonds on the target DNA phosphate backbone (Figures 1 and 3). Because the Cas protein isn't responsible for recognizing the target DNA, it doesn't need any alterations for each unique target sequence. Changing the sgRNA is more affordable and substantially easier than altering proteins, which makes CRISPR/Cas, for all practical purposes, a more versatile tool than either ZFN or TALEN systems. Furthermore, one major advantage of CRISPR/Cas9 is its potential for simultaneous editing at multiple genetic loci by simply transfecting multiple sgRNA molecules into a target cell[1]. It is not hyperbole to proclaim CRISPR-Cas9 as an indispensable, state-of-the-art system in biochemical research with immense potential for translation into the clinic.

Since the advent of CRISPR-Cas9, clinical trials involving some degree of genetic modifications have accelerated remarkably. Per Hirakawa et al., only one clinical trial involving genetic modification was registered in 2009 and two or fewer such trials were registered per year from 2010 through 2015. In 2016, three years after the CRISPR-Cas9 system was described as an efficient gene-editing tool, the number of clinical trials utilizing genome editing technology increased to ten. There were another ten such new trials registered in 2017, and 13 registered in 2018. Additionally, it is noteworthy that ZFN's had been the genetic modification technique of choice prior to 2016 but since then, CRISPR-Cas9 and TALEN gene-editing techniques have dominated the field[5].

While the outlook for CRISPR/Cas in the clinical setting is certainly promising, researchers are still studying and testing this relatively young genetic tool. Safety and efficacy determine the

viability of any treatment, and they are doubly important in gene therapy, as any genetic alterations created, beneficial or deleterious, will become permanent fixtures in all affected cells and their progeny. If reasonable safety can be demonstrated, the effectiveness of CIRSPR/Cas relative to other available treatments will further inform whether the risk:reward ratio favors this new gene-therapy technique. In short, how would a CRISPR/Cas therapy improve upon or augment existing treatments for diseases, and what progress has been achieved in making CRISPR/Cas a clinical reality? Additionally, as this technology is still in its relative infancy, what known limitations currently inhibit CRISPR/Cas utilization as a clinical therapy?

Background:

Infectious Disease

Despite a concerted shift towards antibiotic stewardship in healthcare, with emphasis on responsible prescribing practices, antibiotics continue to be overprescribed. The development of new antimicrobial chemicals, meanwhile, has unfortunately stalled[12]. As a gene-targeting tool, successfully ablating antimicrobial resistance (AMR) genes could make CRISPR/Cas a vital instrument in counteracting the dangers of antimicrobial overprescribing and reinstating susceptibility to entire strains of microbes, and recent findings have demonstrated the possibility of CRISPR technologies aiding in the ongoing fight against drug-resistant bacteria in medicine.

The methodology by which CRISPR/Cas could curb the spread of bacteria exhibiting antimicrobial resistance (AMR) is straightforward: design an AMR-targeting sgRNA and administer it, along with the Cas enzyme, into the appropriate resistant bacterial population to re-sensitize the strain to antibiotics. Bikard et al. first described attempts to disrupt AMR genes via CRISPR/Cas in strains of methicillin-resistant Staphylococcus Aureus (MRSA) and tetracycline-

resistant Staph Aureus. Previous research indicated that lytic bacteriophages may be a potent strategy against drug-resistant bacteria. But lytic phages, which kill bacteria en masse, create a selective stress that almost invariably generates an escape mutation in the targeted bacteria; as a result, the mutated bacterial population ultimately rebounds, and the phage lethality decreases against the new mutant bacterial strain[10]. Moreover, there is a real concern that the phage may also target normal flora that are vital to the host's normal physiologic functions.

To investigate CRISPR/Cas efficacy for destroying AMR genes, Bikard et al treated tetracycline-resistant bacteria with a CRISPR/Cas construct targeting the Tetracycline-resistance gene, using a phagemid vector for delivery; a phagemid is a plasmid enveloped within a bacteriophage lipoprotein capsid. This system demonstrated selective in vitro cell death among virulent bacteria with AMR genes for Tetracycline, within a mixed population (Figure 4), and produced no evidence of significant death among avirulent bacteria. These findings support this initial proof-of-concept experimentation that phagemid packaging of anti-AMR CRISPR/Cas could induce cell death among drug-resistant virulent bacteria while sparing normal flora. Using a phagemid as the vehicle for CRISPR-Cas delivery is not without its own drawbacks and limitations, and researchers noted that the "main obstacle to translation of this technology into a viable therapeutic is the efficient delivery of the Cas9 and its RNA guide(s) into bacterial cells" in vivo[13].

Bikard et al argue the advantages of CRISPR/Cas over lytic phage therapy, but recent publications describe a new hybrid model which hinges upon temperate phages (non-lytic) imbued with a sgRNA-CRISPR/Cas cassette targeting AMR genes. This hybrid phage-CRISPR/Cas system could combine the best of both individual strategies while also mitigating their shortcomings. Such a Phage-Derived CRISPR/Cas (PDC) system would theoretically have

minimal risk for off-target effects against a patient, as bacteriophage exclusively target microbes, not eukaryotic cells, and would spare the avirulent normal flora microbiome.

Liu et al., who studied the possibility of a PDC system featuring a CRISPR/Cas cassette delivered by temperate bacteriophage, published encouraging results for combatting antimicrobial resistance as well. They reported successful incorporation of a CRISPR/Cas cassette into a phage vector, and a follow-up experiment demonstrated efficient eradication of a kanamycin resistance gene within bacteria following treatment with the PDC construct. Following destruction of the kanamycin-resistance gene, previously-resistant *E. coli* bacteria demonstrated re-sensitization to kanamycin. In total, nine *E. coli* host strains were transformed with a PDC construct (targeting kanamycin resistance genes) to test the efficacy of the modified phage to eliminate resistance plasmids. Bacterial re-sensitization to kanamycin was confirmed in all strains via genetic screening after treatment with the PDC construct, followed by observance of in vitro bacterial death upon treatment with Kanamycin to complete this method of Phage-delivered Resistance-Eradication with Subsequent Antibiotic (PRESA) treatment. No subsequent resistance to the antimicrobial agents or to the phage delivery system was observed[10].

To investigate this PRESA strategy in vivo, immunodeficient mice were inoculated with a kanamycin-resistant *E. coli* strain on their skin and intestinal surfaces. Twelve hours following PDC treatment of skin-inoculated mice, only 6.67% bacterial cells isolated from the infection site retained a functional copy of the kanamycin resistance gene, markedly lower than the 98.33% antimicrobial gene retention rate observed in the control group (Figure 5). The intestinally inoculated mice treated with the PRESA method also exhibited significant destruction of the antimicrobial gene. The intestinal *E. coli* exhibited persistent kanamycin re-sensitization throughout the time course of the experiment. Again, no resistant mutants to the

PDC or PRESA methods were observed. This novel PRESA strategy efficiently induced antimicrobial re-sensitization with subsequent bacterial death upon kanamycin treatment and yielded no new resistant mutants[10].

Destruction of resistance genes is arguably as important as killing the virulent bacteria that utilize them, because resistance genes are most often transferred between microbes via circular plasmid DNA, and don't incorporate into the host microbe's chromosomes. So, even if a microbe is killed, its AMR genes may persist and transform a naïve bacterium into a resistant strain. Enzymatically active CRISPR/Cas riboproteins with isoforms Cas9 and Cas3 had previously failed to demonstrate any significant endonuclease activity against bacterial plasmids[14].

Complexes featuring the Cas13 isoform, however, fulfill a different niche role in secondary bacterial immunity: while Cas9 and Cas3 proteins can only target either free floating bacteriophage RNA or chromosomal phage genes, CRISPR-Cas13 complexes can create DSB's within both chromosomal and plasmid DNA[14]. Evolutionarily, this activity of Cas13 helps a microbe defend against temperate bacteriophage genes that have, by chance, integrated into plasmid DNA. So, while Cas9 and Cas3 can only disrupt chromosomal phage DNA, or actively invading phage DNA/RNA, Cas13 can destroy targets on bacterial plasmids in addition to bacterial chromosomes. Cas13 therefore could exhibit more-widespread activity for neutralizing AMR genes.

In their recent paper, Kiga et al (2020) evince the potential of CRISPR/Cas to combat drug-resistant bacteria both in vitro and in vivo with a CRISPR sgRNA targeting plasmid-bound AMR genes in *E. coli*. Their published findings demonstrate 1) in vitro Cas13-mediated DSB induction in plasmids featuring the bla_{IMP-1} AMR gene conferring carbapenem resistance, 2) successful packaging of the CRISPR-Cas13 construct within a phage capsid for delivery into bacterial

cytosol, 3) Bactericidal activity of the bla$_{IMP-1}$ targeting CRISPR-Cas13 construct in vitro, 4) bactericidal activity of the same CRISPR-Cas13 construct in vivo. The lattermost finding was achieved by inoculating larvae of the wax moth *Galleria mellonella* with carbapenem-resistant *E. coli*. Carbapenem resistance was conferred on *E. coli* via a plasmid-bound bla$_{IMP-1}$ gene, and administration of a CRISPR-Cas13 construct targeting the bla$_{IMP-1}$ gene was strongly associated with increased larvae survival following subsequent carbapenem treatment (Figure 6).

Cancer and Immunotherapy

Cancer is essentially the unchecked growth of a person's own cells. Our genome encodes numerous proteins for preventing uncontrolled growth and for recognizing and eliminating cells that are exhibiting cancerous growth behavior. If a protein functions in intercellular communication, cell-cycle regulation, DNA repair, anti-tumor immune response, or some other anti-cancer role, a mutation causing deviant activity could permit cancerous growth. Mutations in one of two broad genetic categories in particular – oncogenes or tumor suppressor genes (TSG) – can drive cancer. Wild-type oncogene proteins promote cell growth and mitosis, but are subject to careful control, so mutations that yield constitutively active oncogenes could drive tumorigenesis. Conversely, TSGs suppresses growth and mitosis, especially when DNA damage is detected, so a mutant TSG could fail to curb inappropriate cellular growth[16].

There are several important milestones that a nascent cancer must achieve before it is considered malignant or concerning. Two major steps in cancer development involve escape from host immune surveillance and attaining immortality[16]. Because mutations induce these steps towards tumorigenesis, genetic alterations that remove or nullify these via CRISPR/Cas is a promising option for the future of cancer therapy.

The natural obstacle preventing cancer cell immortalization involves chromosomal telomeres, a unique structure in eukaryotic chromosomes that is key for chromosomal integrity. Telomeres are the distal-most ends of chromosomes; they feature numerous repeats of a nucleotide motif (5'-TTAGGG-3') and have a 3' overhang which performs a strand invasion into an upstream telomere segment to provide a protective cap on the ends of chromosomes[16] (Figure 7). With every mitotic cycle, the telomeres become shorter and shorter until, after approximately 40 cycles, the telomeres are too short for cell viability, causing the cells to enter either senescence or experience an event called "crisis". Senescence, the much more common pathway, is characterized by both cell cycle arrest and the end of cellular division for the senesced cell. When short telomeres incite crisis, the chromosomes lose their protective telomeric cap, chromosome fusions occur, and irreparable genomic instability results, effectively ending the cell's mitotic potential and frequently causing apoptosis[16].

A common means of avoiding or overcoming either senescence or crisis involves activating the telomere-lengthening enzyme Telomerase. Telomerase is a ribonucleoprotein with two important subunits: an RNA subunit encoded by the TERC gene, and a reverse transcriptase (RT) which is encoded by the TERT gene. The RT subunit uses the RNA subunit as a template for elongating telomeres. The enzyme is active only in germ-line cells – sperm and ovum – in humans. As TERT is suppressed in non-cancerous somatic cell, and normal somatic cells' telomeres shorten with each mitotic cycle as a result, TERT could be considered an oncogene that drives cancer proliferation when constitutively active by promoting immortalization[17].

Current data suggest that 80-90% of cancer cells fail to repress TERT, allowing for telomere elongation and subsequent avoidance of senescence and crisis[17]. As a common feature of cancer cells, specific TERT/telomerase targeting is a potential target for cancer therapy. It is worth

noting that an alternative pathway for escaping senescence and crisis exists which involves spontaneous NHEJ-mediated fusion of uncapped telomeres, but this is much less common and involves a complex interplay between DNA repair proteins[8,16]. Telomerase, conversely, is a single enzyme that, due to its ubiquity in cancer cells, is a prime target for anti-cancer CRISPR/Cas therapy.

Wen et al. report successful disruption of the TERT gene via CRISPR-Cas endonuclease activity, which was achieved in vitro with no off-target mutations detected. Additionally, in vitro growth was significantly stymied by CRISPR/Cas-mediated mono-allelic TERT knockout, demonstrating that inducing TERT haploinsufficiency may be adequate for preventing telomere elongation and could indeed serve as a powerful method for slow tumor growth/progression. To investigate the effect of TERT haploinsufficiency in vivo, immunodeficient mice underwent hind leg grafting with Hela cells: the left hind leg of each mouse was grafted with Hela WT cells (WTPE) and the right hind leg was grafted with Hela cells with sequence-confirmed TERT haploinsufficiency. The WTPE group developed tumor growth in all animals, while grafting of the experimental TERT haploinsufficient cells failed to develop any tumor growth in all subjects. These results further demonstrate the potential of CRISPR/Cas-based therapies to suppress tumor growth by inducing TERT haploinsufficiency (Figure 8).

In addition to immortalization, long-term survival of a neoplasm also depends on avoiding detection and destruction by the host immune system. T-cells, specifically, are a major force in defending against growing cancers, and patients whose cancers are poorly detected by host T-cells are associated with worse outcomes. Harvesting T-cell progenitors, genetically augmenting them to recognize cancers, and subsequently infusing the altered T-Cells is a promising avenue of CRISPR/Cas research. Chimeric antigen receptors (CAR) are cell-surface proteins that have

been engineered to recognize antigens that are commonly associated with tumors; they are termed 'chimeric' because they exhibit a tandem of antigen recognition and T-cell activating functions. T-cells with CAR proteins (termed CAR-T cells) have repeatedly demonstrated promise as a therapeutic adjunct, but engineering them is a difficult process with significant hurdles to overcome before this theoretical experimental treatment can enter the clinic[18,19].

Most CAR-T clinical trials practice transfusion of autologous cells that had previously been harvested and genetically edited. This ex vivo practice is both time consuming and expensive, two factors that could limit the potential of CAR-T therapy. A hypothetical allogenic line of CAR-T cells, derived from one donor and readily available to patients "off the shelf", would potentially lower costs, decrease time between diagnosis and treatment, and almost certainly benefit more people than a practice of autologous modified cells alone. The potential of Graft Versus Host Disease (GVHD) is preventing such an off the shelf CAR-T cell therapy from becoming a reality; innate proteins of the allogenic CAR-T cells' host could either recognize the recipient as foreign, or the recipient's immune system may recognize the donor's innate antigens[18], and either manifestation of GVHD would severely undermine the prospect of an "off the shelf" allogenic CAR-T cell therapy.

The primary feature of developing CAR-T therapy, as discussed, involves equipping cells with a gene that will improve a patient's tumor suppression capabilities, but minimizing problematic antigens within the donor T-cells also appears to be a necessary step for achieving allogenic CAR-T cell therapies. As mentioned above, a CRISPR/Cas mediated DSB can ablate genetic function when the DSB is followed by error-prone NHEJ DNA repair. It appears that CRISPR/Cas, in addition to facilitating the addition of a tumor-recognizing CAR gene, may also be necessary for minimizing GVHD by knocking out endogenous cell-surface proteins[18].

Immunotherapy with CAR-T cells is further limited by data demonstrating little to no effect in treatment of solid tumors. A complicated interplay of inhibitory factors constituent to the tumor microenvironment are likely partially responsible for this low observed efficacy, but CAR-T cells, like all T-cells, can be especially hampered by a process called "exhaustion". During T-cell exhaustion, the intercellular functions of T-cells are depressed, while expression of inhibitory receptors simultaneously increase, further diminishing T-cell function. Exhaustion ultimately causes apoptosis, and a major mediator of this exhaustion-apoptosis sequence is the Fas gene. Preventing either CAR-T exhaustion or cell inhibition by inhibiting Fas function could expand the utility of CAR-T cells if/when they are ever utilized clinically[18]. In short: multiple genetic alterations are required to attain "off the shelf" CAR-T cells.

Ren et al. reported the successful ablation of genes encoding cell surface proteins CD3 and HLA-1, as well as the apoptosis-promoting Fas gene, with a one-shot CRISPR transfection into CAR-T cells. These alterations reduce the potential for GVHD, while simultaneously prolonging survival of the CAR-T cells, thereby extending their therapeutic window. Studies of the triple-ablated cells both in vitro and in vivo demonstrated increased survival relative to wild type (WT) cells, including when apoptosis was chemically induced[18].

Genetic diseases

Because of their ease of access and minimal invasiveness, the integumentary and hematologic systems are an excellent platform for testing/monitoring experimental CRISPR-Cas therapies. Epidermolysis Bullosa (EB) is a rare dermatologic condition that yields skin blisters from even minimal trauma or fiction, with mucous membranes and other organs being involved in some subtypes. Over 20 different genes have been implicated in EB, and varying combinations of one

or multiple genes have been identified in EB patients[20,21,22]. The epidermolysis bullosa phenotype can manifest as a result of mutated genes that produce dysfunctional proteins, and the causative mutations can display variable penetrance, with some mutated genes showing dominant expressivity and others showing recessive expressivity[21,22]. Previous findings had demonstrated that grafting autologous skin exhibiting normal phenotypic integrity could provide symptomatic relief of EB[20], but this approach is not feasible for a person with extensive EB.

Recessive dystrophic epidermolysis bullosa (RDEB) is caused by loss-of-function mutation at a single locus in the gene encoding type VII collogen (*COL7A1*; C7). Prior attempts at genetic modification via ZFN and TALEN to treat EB were in various stages of development prior to the advent of CRISPR-Cas9, which is now considered the more attractive modality for investigating EB gene therapy. Mutant cells could be treated by CRISPR/Cas with an ex vivo approach of removing autologous stem cells, correcting the causative mutation in a laboratory, and grafting the modified cells onto a patient to hopefully introduce WT function. Successful and efficient restoration of a functional C7 gene for collagen was indeed reported in human keratinocytes via CRISPR/Cas treatment (Figure 9). The corrected keratinocytes were subsequently bio-engineered into skin constructs, which in turn were grafted onto immunodeficient mice. After successful graft incorporation, the grafted regions were subjected to rigorous in vivo stresses and skin regeneration testing [21].

The proof of concept reported by Bonafont et al. was essentially reaffirmed, albeit while also showcasing the remarkable versatility that CRISPR-based gene editing affords. The above findings report successful grafting of human keratinocytes, but Jackow et al. successfully corrected C7 and subsequently grafted induced pluripotent stem cells (iPSC), again onto immunodeficient mice. Successful correction of iSPC's, which exhibit less cellular

differentiation and consequently are much more versatile, could mark an important milestone in translating CRISPR-based technologies into the clinic. Normally, iSPCs manipulation is associated with greater genetic instability relative to manipulation of highly differentiated cells, but utilization of a high fidelity Cas9 enzyme yielded no discernable off-target effects[22]. The resulting iPSC grafts provided long-term safety and efficacy in the immunodeficient mouse models. These genetic corrections and tissue engineering techniques could represent a therapeutic option by which patients attain long lasting, even permanent relief from their non-healing wounds in RDEB. Overall, the most recent studies of this kind offer compelling evidence for the efficacy and safety of a novel CRISPR/Cas9-based ex vivo gene-editing approach for clinical treatment of multiple EB subtypes.

Sickle Cell Disease (SCD) and Beta-Thalassemia are the clinical result of mutation in the Hemoglobin B (HBB) gene, which impairs the functionality of the b-globin subunit in adult hemoglobin. In SCD, the mutations result in sickle-shaped red blood cells (RBC); in Beta-Thalassemia, the mutations yield decreased hemoglobin production on RBCs. Consequently, Beta-thalassemia patients can experience fatigue, bone deformities, and people with SCD suffer from significant morbidity in the form of severely painful sickle cell crisis attacks and multi-organ damage (due to increased coagulability of sickled RBCs); both conditions are associated with early mortality. Allogenic transplantation of Hematopoietic Stem Cells (HSC) can be curative, but also carries the risk of severe toxicity, especially if harvested from a donor with sub-optimal histocompatibility[23].

HBB mutations can theoretically be “corrected” and returned to wild-type by CRISPR-Cas9 editing, but it requires introducing an exogenous donor template and relying on the less-prevalent HDR pathway, which, again, is not ubiquitously active throughout the cell cycle. Other studies

have shown that elevated fetal hemoglobin (HbF) production is associated with reduced morbidity and mortality in both SCD and b-thalassemia, and prior research had demonstrated induction of HbF production in adults by nullifying regulatory transcription factors (HBG1/HBG2) that repress HbF production. Métais et al. elected to pursue this promising avenue of knocking out the HbF inhibitor for treating SCD and Beta-thalassemia patients because it utilizes constitutively-active NHEJ pathway following Cas-mediated DSB to effectively inactivate the HbF transcription inhibitor.

A CRISPR-Cas9 sgRNA was designed to target a specific sequence (the BCL11A consensus motif) in the HBG1/HBG2 gene promoters for the purpose of ablating inhibitory effect of HBG1/HBG2 on the HbF genetic locus, thereby increasing HbF expression. Human CD34+ hematopoietic stem and progenitor cells (HSPCs) were treated with Cas9:sgRNA-1 RNP complexes via electroporation to ablate the HSPCs' HBG1/HBG2 promoters. In vitro analysis of edited 34+ HSPCs following electroporation confirmed that gene editing of the HBG1 and HBG2 gene promoters raised the percentage of both F-cells and HbF protein significantly, demonstrating viability and efficacy of NHEJ-mediated knock-out of HfB regulator gene HBG1/HBG2, resulting in increased HfB expression[23] (Figure 10).

A nearly identical approach has recently been reported in the news media, in which a woman has entered into a clinical trial for ex vivo modification of her own HSPC cells to induce production of fetal hemoglobin HfB, followed by autografting the modified cells for experimental treatment of her SCD. While the results of this clinical trial have not yet been published, the recipient of the therapy has participated in interviews with the media, and she reports subjective symptomatic improvement. Additionally, her physician and research team report reduced morbidity, improved hemoglobin concentration on blood diagnostics, and

increased HfB production. A biopsy of this SCD subject's bone marrow cells revealed that 81% of the collected cells contained the intended genetic change targeted by CRISPR/Cas to induce fetal hemoglobin production. This finding indicates that edited cells have survived and are demonstrating long-term expression of HfB. Of note, the clinical trial includes subjects who have undergone this procedure for treatment of Beta-Thalassemia as well, though no data or anecdotal reports have been published regarding these cases[24].

Materials and Methods:

The research and reviews discussed herein were found primarily using the following search terms: CRISPR, Cas, Cas9, clinical trials, clinical therapy, Stem cells, iPSC, Epidermolysis Bullosa, CAR-T cells, antibiotic, antimicrobial, resistance, Sickle Cell Anemia.

The search engines and/or websites utilized in researching this review included: NCBI, also known as PubMed (ncbi.nlm.nih.gov/), Nature Journals and Communications (nature.com), Elsevier (elsevier.com), Google Scholar (scholar.google.com), the JAMA Network (jamanetwork.com), and the Augsburg University Library (lib.augsburg.edu).

Of note: several articles discussed herein were suggested by a website based on previously viewed pages, and not intentionally sought or discovered via the above search terms. Additionally, full access to several publications were available for free, but most were obtained via the Augsburg University Library.

Discussion:

Ever since the power of CRISPR/Cas was first realized in the laboratory, there has been a growing hope that its efficient, cost-effective power for altering genes could directly benefit patients in the clinical setting. Treatments for diverse categories of disease discussed herein – infectious, genetic, and neoplastic diseases – could one day include some form of CRISPR/Cas therapy. That hope, while certainly ambitious, is not baseless. While only one subset of diseases discussed here is explicitly about genetic diseases, multi-drug resistant (MDR) infections and cancers are also dependent on their underlying genetics. AMR genes and the oncogene-TSG tandem enable bacteria and cancer, respectively, to evolve into life-threatening illnesses. The possibility of unleashing a potent gene editing system like CRISPR/Cas against the genes that potentiate these diseases has inspired research across virtually every specialty in medicine. The publications discussed herein represent a small fraction of the encouraging progress achieved thus far, progress that will, hopefully, one day make CRISPR/Cas a viable clinical instrument.

Antimicrobial resistance

The above studies, which focused on silencing AMR genes, show the ability of CRISPR/Cas to 1) target virulent strains of bacteria while sparing avirulent species, 2) selectively target AMR genes on chromosomes as well as transferrable plasmids, and 3) re-sensitize antimicrobial-resistant microbe strains in vivo. While these methods are not yet ready for clinical practice, the results discussed above are nonetheless promising because, as previously mentioned, development of novel antimicrobial chemical agents has slowed to a halt, and community-acquired strains of multi-drug resistant strains have been observed in clinical settings.

As a means of slowing and perhaps reversing the spread of AMR genes, CRISPR/Cas antimicrobials are one of the most promising avenues currently being explored. When antimicrobial resistance was first recognized as a growing problem, combination therapy involving multiple antibiotic classes was touted as a new strategy for slowing the spread of AMR genes. In theory, combination therapy could disrupt multiple intracellular mechanisms and compound the drugs' bactericidal effects, possibly making the efficacy of the combination therapy greater than the sum of its parts. However, no improvement in patient mortality was observed when combination therapies were tested. Antibiotic hybrids[25], in which two antibiotics or an antibiotic and an adjuvant are covalently bonded, are currently being evaluated in clinical trials, and are showing promising preliminary results in killing MDR bacteria in vivo. However, the underlying AMR genes remain undisturbed, and it is entirely conceivable that a new spontaneous mutation could confer resistance to hybrid antibiotics.

Per the United Nations, approximately 700,000 people die from drug-resistant bacterial infections annually, and estimates by the UN suggest that the death toll could expand to 10 million annually by 2050. To take a broad view of the future of antibiotics, it is the author's opinion that CRISPR/Cas antimicrobials would yield more sustainable results than a novel antibiotic or a hybrid antibiotic treatment. Certainly, any breakthrough against drug-resistant bacteria is good news, regardless of the source, so relying solely on CRISPR/Cas technology would be imprudent. And bacteria could conceivably develop resistance to any novel antibiotic drug or technique, including CRISPR/Cas-based AMR destruction. But Cas antimicrobial enzymes are fundamentally different from traditional antibiotics, and acquiring resistance to CRISPR/Cas antimicrobials would almost certainly be more difficult than acquiring resistance to

chemical antibiotics. The work by Liu et al. and Kiga et al. specifically report that bacteria, when treated with CRISPR/Cas antimicrobials, failed to produce any escape mutants.

A bacterium must spontaneously mutate to negate a traditional antibiotic in the absence of plasmid-based AMR vectors, but traditional antibiotics are chemically simpler than a comparatively massive Cas enzyme or CRISPR/Cas-encoding plasmid cassette. Furthermore, the limited literature reviewed here mentions several possible delivery systems, whereas traditional antibiotics can only enter through simple or facilitated diffusion. So, eliminating a Cas enzyme or a delivery cassette would be more difficult than negating a traditional antibiotic, and there are multiple methods for delivering the CRISPR/Cas anti-AMR products than traditional antibiotics, so achieving resistance to one delivery may be insufficient for a bacterial population. In short, spontaneously mutating to adapt to CRISPR/Cas is much more complicated for bacteria than negating or remove a small antibiotic. Furthermore, because CRISPR/Cas can be programmed to destroy essentially any AMR gene, the gene effectively dies with the host microbe. Frequently after a bacterium dies, its plasmids remain intact as do the AMR genes they may carry. This allows a plasmid to transfer AMR genes to a new strain repeatedly[26]. This has partially contributed to the rise of MDR bacteria, and CRISPR/Cas-mediated AMR gene destruction is the first system to directly destroy the underlying AMR genes and thereby end their continuous propagation.

In recent years, multiple studies have demonstrated the importance of a healthy microbiome with a diverse normal flora microbe population. One modern example demonstrating the importance of a healthy microbiome involves *clostridium difficile* infections. Antibiotics do not discern between virulent and avirulent bacteria, so a patient completing a course of antibiotics almost certainly will kill a percentage of their avirulent normal flora. When a person's normal

homeostasis within the microbiome is disrupted by mass antibiotic-induced death, bacteria such as *C. Diff* can fill the microbial void, potentially leading to a serious intestinal *C. diff* infection. With that in mind, the findings discussed here that demonstrated antimicrobial CRISPR/Cas activity while sparing the normal flora are encouraging.

Delivering the CRISPR/Cas system in vivo remains a major obstacle for clinical utilization and is currently the subject of ongoing research. Kiga et al. and Liu et al. reported encouraging results demonstrating increased in vivo survival in their wax moth larvae and mice experiments, respectively, but translating that into human populations will absolutely be a challenge.

Cancer therapy

Correcting the mutations driving cancerous growths – or altering the immune system to recognize an evolving neoplasm – are exciting strategies for cancer therapy, and the CRISPR/Cas system is uniquely equipped to develop these strategies into a clinical reality. The novel findings of TERT suppression, and associated failure of tumor cells to grow on immunodeficient mice, is an exciting finding given how prevalent TERT activation is in cancer. Preventing cancer immortalization via telomerase ablation could be a powerful adjunct to, or replacement for, chemotherapy. Deactivating Telomerase in somatic cells in vivo, however, is significantly more difficult than the ex vivo xenograft recounted in this review; future investigations for achieving Telomerase inactivation with an in vivo strategy will almost certainly be explored.

Several studies have been published since 2017 regarding the promise of CAR-T therapy as a new cancer treatment. Ren et al. successfully demonstrated CRISPR/Cas-mediated knock out of CAR-T cell surface proteins that would normally create risk for GVHD (CD3 and HLA-1).

Additionally, a new strategy for creating long-term viability of CAR-T cells post-transfusion was identified by CRISPR/Cas-mediated knockout of the apoptosis-promoting Fas gene, and CAR-T cells demonstrated a longer active lifespan as an in vivo xenograft following successful inactivation of Fas. What's more, they were able to accomplish a triple knockout (CD3, HLA-1, and Fas) in one fell swoop by packaging the CRISPR system with multiple sgRNAs. As more sgRNAs are co-introduced with a Cas enzyme into a cell, there is greater theoretical potential for off-target effects. No such off-target effects were reported, however. Taken together, these findings demonstrate the power, versatility, and efficiency that CRISPR/Cas gene editing affords a researcher, and further illustrate the potential for clinical CRISPR utilization.

Current cancer therapies generally fall into three categories: surgical excision of a tumor mass, radiation therapy, and chemotherapy[15]. These therapies can absolutely be successful individually and in concert, but they are not without drawbacks. Surgical excision involves cutting out as much of a tumor mass as possible, but it's simply impossible to remove every cancer cell in surgery. Radiation Therapy aims to destabilize the genome of a cancer cells via directly exposing a cancerous mass to ionizing radiation, but non-cancerous cells are inevitably caught in the crossfire. Finally, chemotherapy is a systemic infusion of medications that preferentially attack cancer cells, but, once again, non-cancerous dividing/active cells are also damaged. Each method has been honed and improved over time, of course, but their shortcomings remain.

The above studies demonstrate how CRISPR/Cas-based cancer therapy could better discriminate between cancer and healthy cells. As previously mentioned, telomerase is only active in cancerous somatic cells, so disrupting TERT – and by extension, telomerase – would only adversely affect cancer cells. Given how frequently active TERT is found in cancer cells, its

inactivation as described by Ren et al. could evolve into a new therapy against cancer. Similarly, CAR-T cells that selectively target tumor-associated antigens could additionally aid in eradicating cancer cells while minimizing toxicity and side effects to the patient. Moreover, the work to create an "off the shelf" allogenic CAR-T cell therapy could expedite treatment following new cancer diagnosis, and CAR-T cells are currently being developed to specifically target cancer cells alone and minimize damage to healthy cells. Surgical resection, radiation therapy, and chemotherapy will likely always have a place in cancer therapy, but CRISPR/Cas utilization to knockout TERT and hone CAR-T cells could develop into powerful complimentary therapies.

Genetic conditions

The EB research recounted here represent some of the most promising findings for CRISPR/Cas as a therapeutic implement. Since Hirsch et al. first described autografting unaffected dermis to achieve symptomatic relief, the strategy in the field has involved variations of an ex vivo modification strategy, in which incorporation of a functional WT gene replaces the mutant protein in EB patients. Multiple studies since have created EB-corrected skin constructs which were successfully grafted onto immunodeficient mice. Moreover, Jackow et al. demonstrated successful CRISPR/Cas-mediated gene correction in iPSC's. This represents a monumental achievement, as iPSCs and PSCs are versatile and can differentiate into numerous specialized cells. These are promising steps towards clinical reality, but some barriers remain. Successful fusion of the graft onto a human dermal basement membrane has not yet been demonstrated and, as always, ensuring no harmful off-target effects is key. Techniques to refine the ex vivo

correction strategies, or potentially developing new in vivo approaches, are worth exploring for future research.

As previously mentioned, there is currently an ongoing clinical trial in which CRISPR/Cas successfully deactivated an inhibitory transcription factor against fetal hemoglobin to increase serum HbF and relieve SCD symptoms. While no peer-reviewed published data is available, lab findings and the subjective accounts from the subjects and their physicians are encouraging. As reported by NPR, researchers had hoped to achieve 20% HfB hemoglobin expression in studies following the treatment, but early results show the subject consistently expressing HfB over 40%[24]. Of note, the subject has reported that the pain associated with SCD has improved since she underwent treatment in June 2019, and has required less narcotic analgesia for controlling her pain[24]. Fewer details regarding the Beta-Thalassemia patients was available, but they have reportedly required less frequent blood transfusions since undergoing treatment with CRISPR/Cas-altered progenitor cells. If the subjects' symptoms and biomarkers persistently demonstrate improvement in key parameters, such findings would represent a monumental step forward for CRISPR/Cas technology, and exhibit the safety and therapeutic power of CRISPR/Cas gene therapy to the medical community at large.

Current treatments for most genetic diseases currently center on symptomatic treatment and minimizing complications. EB, for example, with its characteristic painful blistering/sloughing of skin and reduced skin integrity, leaves a person vulnerable to infection and chronic pain, so current therapies include prophylactic antibiotics, topical skin treatments, and skin grafting in appropriate candidates. Similarly, SCD and Beta-Thalassemia treatments involve controlling and minimizing symptoms with medications and blood transfusions.

Gene therapy involving permanent correction of a mutation at the genetic level would achieve chronic expression of the corrected gene, which would, in theory, be curative for the genetic condition in all treated cells and their progeny. There are genetic diseases for which organ or bone marrow transplants can effectively be curative, but numerous incurable genetic diseases remain. This promise of gene therapy explains why CRISPR/Cas has received so much attention in both the laboratory and the public consciousness. Effective and safe gene therapy in a human subject, no matter what system, is an unmitigated success, and ZFN and TALEN systems have both demonstrated some efficacy in gene therapy clinical trials. But relative to ZFN and TALEN gene-editing techniques, CRISPR/Cas is more affordable, more easily programmable, exhibits superior specificity and, as reported in the above literature review, has shown great promise with in vivo experiments and in clinical trials. In short, CRISPR/Cas achieves higher fidelity editing with lower costs and appears situated as the primary candidate for creating clinically relevant gene therapies going forward.

Future Directions

The practical outlook of CRISPR/Cas therapy is promising, but several hurdles remain, including achievement of in vivo delivery and minimizing off-target effects. Initially, there was promise that viral delivery of CRISPR/Cas constructs may enable extensive in vivo delivery, like the bacteriophage or phagemid delivery systems described here for CRISPR/Cas delivery into bacteria. Progress has also been made with Ribonucleoprotein delivery, in which the transcribed CRISPR/Cas system is delivered in the form of mRNA, may be the best vector for continued pursuit of clinical CRISPR applications as they can be modified to recognize tissue-specific cell surface proteins, potentially enabling efficient targeting of a cell type in vivo[11]. To minimize off-

target effects, several high-fidelity modified Cas isoforms have been described and utilized in human cells in vitro, with more modified Cas variants in development[27]. However, there are no reports available describing any ex vivo-type experiment like the studies described here.

CRISPR/Cas has entered the zeitgeist in the research community because, in addition to its cost-effectiveness, it yields fewer unwanted indels relative to ZFN and TALEN editing techniques. However, the risk of off-target genetic effects still requires mitigation before CRISPR/Cas-based gene therapy utilization can be expanded[5,28].

Conclusions:

CRISPR/Cas has, in a few short years, developed from a little-studied microbial curiosity into an indispensable scientific tool that is driving discovery and creating new methods of treating disease. The ease with which CRISPR/Cas can be reprogrammed enables it to target any desired DNA sequence, meaning that genetic diseases, infectious diseases, and cancer can all theoretically be treated by well-controlled alterations of the underlying genes that cause, enable, or fail to prevent a given disease. That is why the scientific community is so excited about CRISPR/Cas: It has the potential to treat diseases that appear categorically different, because their root causes are genetic in nature. Clinical trials involving gene alterations have increased substantially since CRISPR/Cas was first described as an efficient gene editor, and scientists are consistently finding new ways to utilize this powerful system.

Before it can enter the clinic in earnest, however, ethical ramifications and considerations of CRISPR/Cas require thorough discussion and debate. The studies and therapies discussed throughout this report have centered on altering genes of somatic cells, the results of which are not passed on to subsequent generations. But the hypothetical editing of germ-line cells could

potentially be retained in all subsequent progeny. Additionally, it is entirely possible that a child with a genetic disease, perhaps EB or Duchene Muscular Dystrophy, may benefit from early correction of the disease-causing gene. There are numerous ethical dilemmas to consider before rolling out CRISPR/Cas. Is it ethical to edit the genome of nascent embryo? In what conditions, if any, are genetic modifications appropriate in a patient who has not reached the age of consent, or who is unable to consent? When is gene editing cosmetically indicated, if ever? And of course, as an issue of justice, how do institutions minimize or, ideally, eliminate socioeconomic barriers that would prevent people who are impoverished or otherwise disenfranchised, from receiving gene therapy?

These questions are not just hypotheticals. In 2018, an experiment by He Jiankui out of Southern University of Science and Technology in Shenzhen, China reported the first CRISPR/Cas-mediated editing of human embryos[29]. To briefly recap the study's purpose, the CRISPR/Cas alteration reported by He was meant to reduce the embryos' risk of contracting HIV, as the eggs were extracted from an HIV positive woman prior to in vitro fertilization, and the woman was set to have the embryo transferred into her uterus. The edited embryo ultimately progressed normally and developed into two healthy females that were born at full term. There were clear issues with the experiment, and no definitive evidence exists demonstrating that He actually succeeded in editing the targeted genes. The experiment was almost universally decried by the scientific community as a reckless exercise, but the outcry doesn't change the irreversible germ-line mutations that He supposedly achieved. Permanently altering the genes of two girls was unethical, impulsive, and irresponsible, but none of these ethical concerns stalled He's research. The flaws and failures of He's experiment show that, without proper involvement and guidance of bioethicists, the public won't readily trust CRISPR/Cas as a powerful tool for good.

The CRISPR/Cas system appears likely to yield novel therapies, and could aid in treating diseases discussed here, as well as some not previously considered in this report. The studies discussed herein showcase the diverse panoply of diseases that are candidates for CRISPR/Cas-based therapy, despite having fundamental differences in etiology and disease course. As a research tool, CRISPR has proven itself to be a modern scientific essential, and the findings reviewed here, in which a diverse array of diseases are considered candidates for CRISPS/Cas treatment, provide promise for the future of therapeutic CRISPR/Cas utilization.

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Supplemental Figures

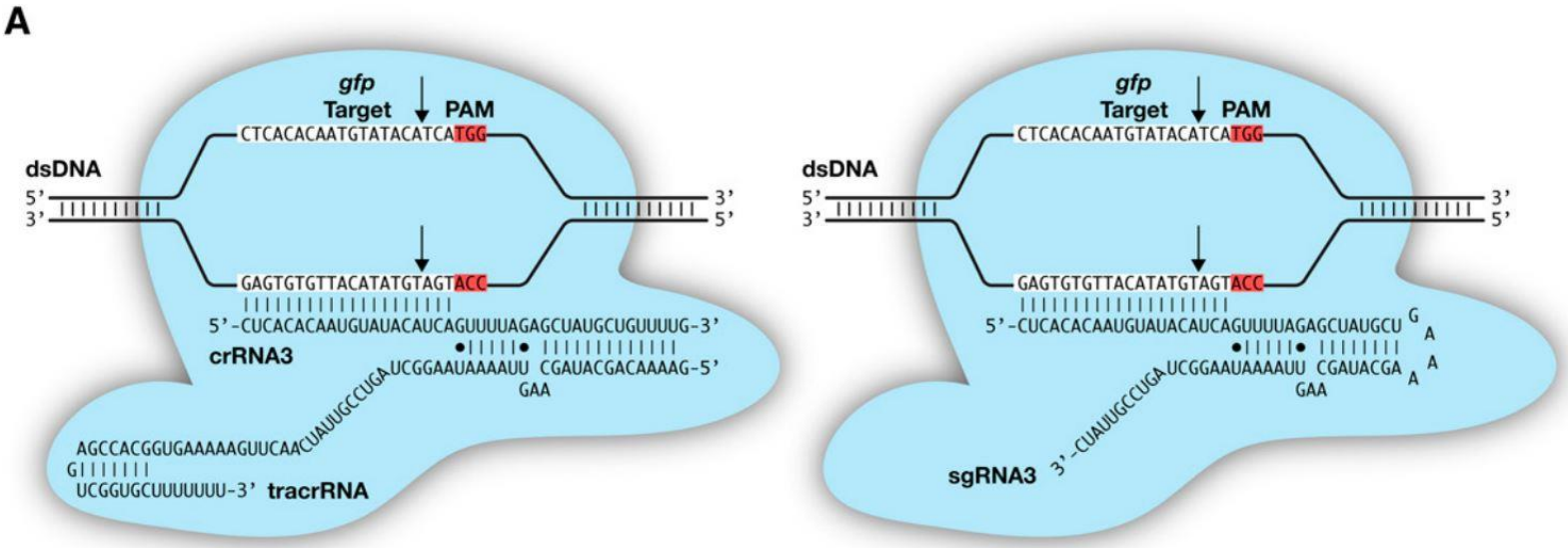


Figure 1: A structural schematic of CRISPR/Cas riboprotein. The Cas enzyme is the large blue object, dsDNA exit either side of the upper half. Within the Cas enzyme, DNA unwinds and one strand hybridizes with complimentary guide RNA. Guide RNA is comprised of either crRNA and tracrRNA in tandem (left) or a sgRNA (Right) which essentially combines the crRNA and tracrRNA with a hairpin loop structure. The target gene on the DNA sequence is highlighted in white within the cas protein, and the site of Cas-mediated DSB induction is denoted with black arrows.[3]

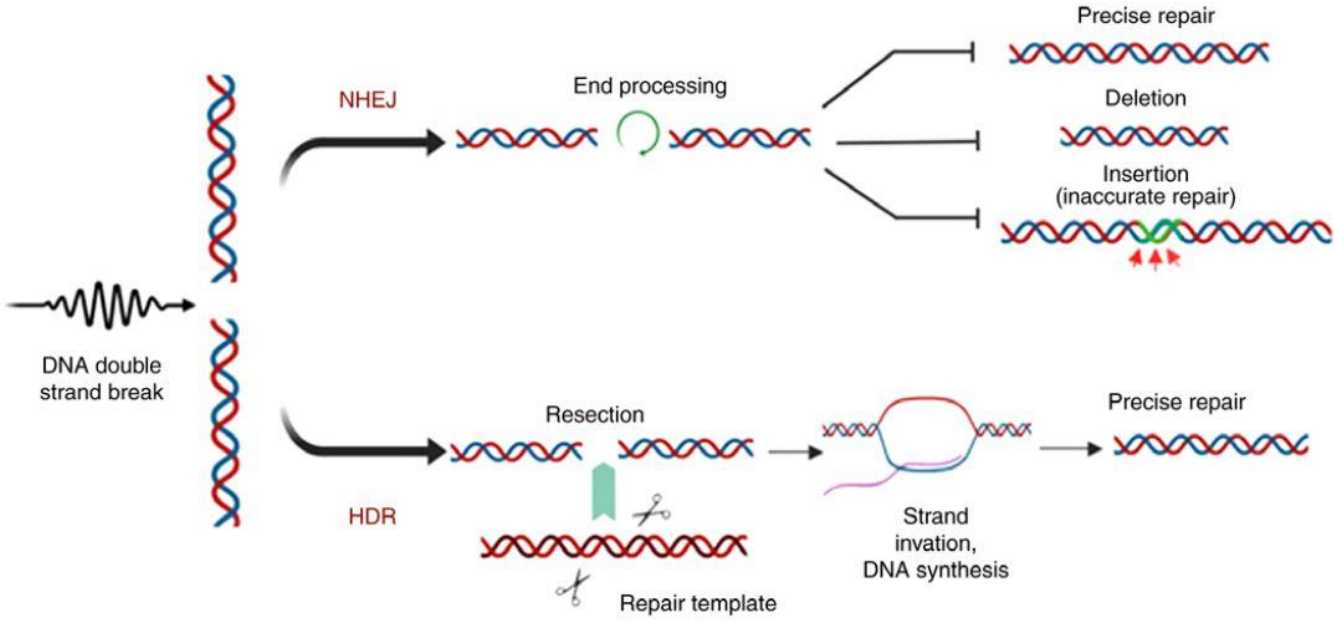


Figure 2: Innate DNA repair mechanisms that guide gene editing. Formation of a DSB to initiate DNA repair by either NHEJ to yield indel events (top path) or HDR, which uses a template DNA strand for repair (bottom path).[28]

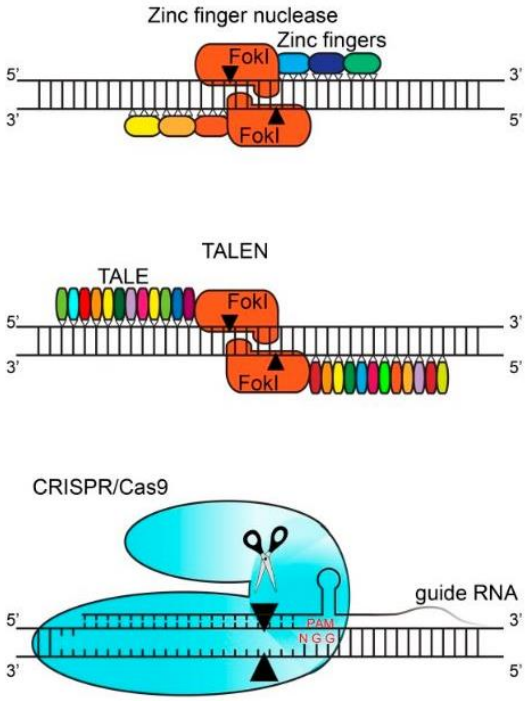


Figure 3: Structures of ZFN, TALEN, and CRISPR/Cas nucleases. Both ZFN and TALEN feature a Fok1 restriction enzyme that induces DSBs within non-specific nucleotide sequences, leaving behind a 5' tail. Specificity for ZFN-mediated cleavage is accomplished with unique Zinc Finger protein motifs, that bind nucleotides triplets around the desired cut site. A unique TALE protein sequence likewise guides Fok1 to the desired sequence in the TALEN system by binding single nucleotides. Unlike TALEN and ZFN systems that utilize protein-based guides, a guide RNA sequence enables Cas-mediated DSBs in the CRISPR/Cas system.[11]

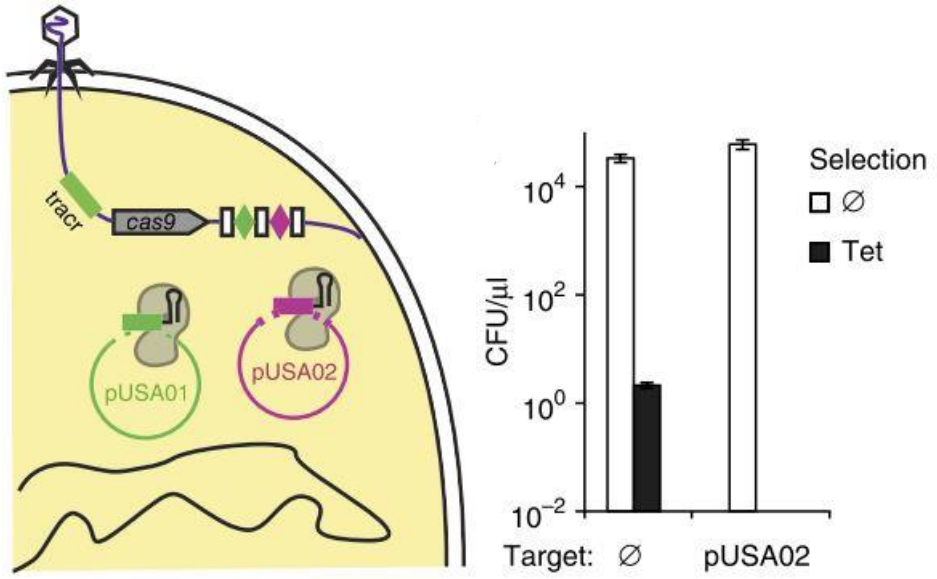


Figure 4: Delivering a phagemid-based anti-AMR CRISPR/Cas construct. The phagemid-packaged CRISPR/Cas sequence is programmed to target two distinct plasmids (pUSA01 and pUSA02) simultaneously. pUSA02 confers Tetracycline resistance. Bacterial colonies were plated on either a normal agar or on a tetracycline (Tet) selection medium after treatment with a non-targeting CRISPR/Cas construct or a CRISPR/Cas targeting the tetracycline resistance plasmid pUSA02.[13]

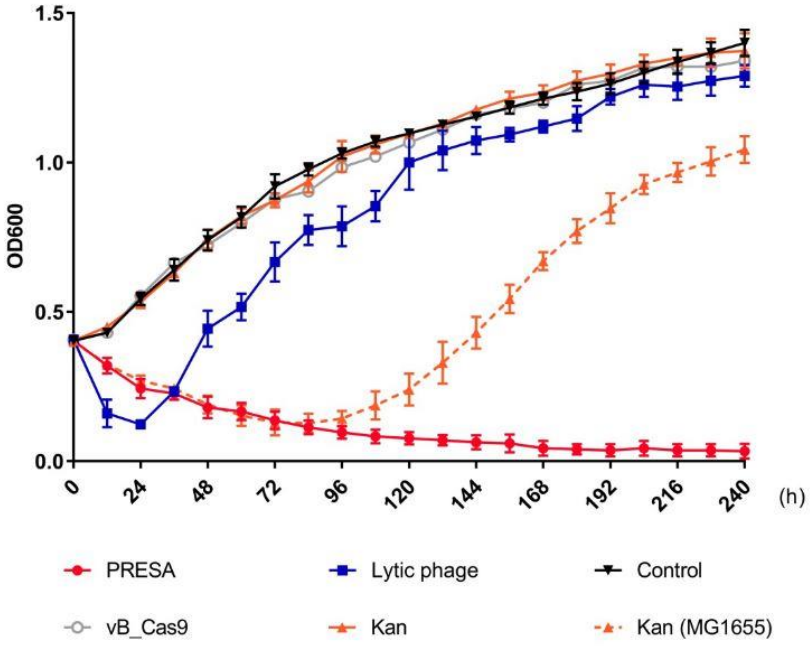


Figure 5: solid lines represent the growth curves of the Kanamycin-resistant strain treated with: PRESA strategy, Lytic Phage, CRISPR/Cas targeting Kanamycin resistance alone (vB_Cas9), and Kanamycin (Kan), as well as an untreated control. Additionally, some plates were inoculated with both two *E. Coli* strains, one with Kanamycin resistance and one lacking kanamycin resistance (MG1655), which is represented as a dotted orange line.[10]

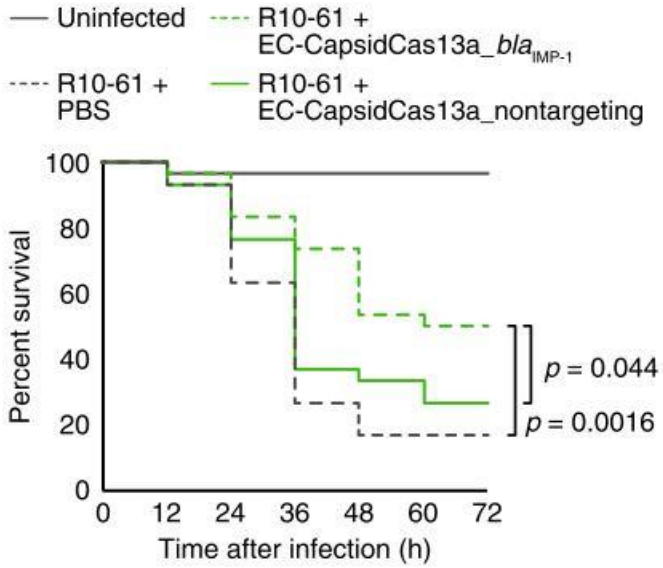


Figure 6: Therapeutic effect of CRISPR/Cas using in vivo *Galleria Mellonella* infection model. Administration of anti-resistance CRISPR/Cas (EC-CapsidCas13a-blaIMP-1) into *G. Mellonella* larvae infected with Carbapenem-resistant *E. Coli* isolates significantly improved host survival when compared with both a non-treatment group and a non-targeting CRISPR/Cas construct treatment group.[14]

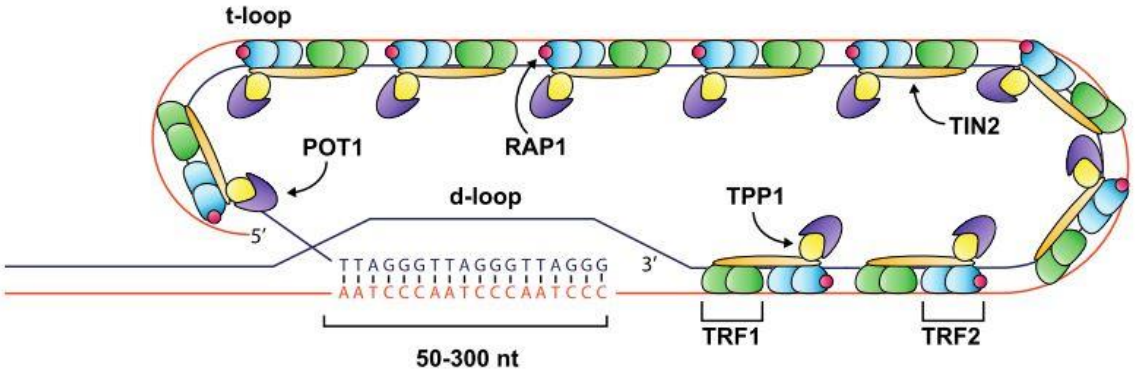
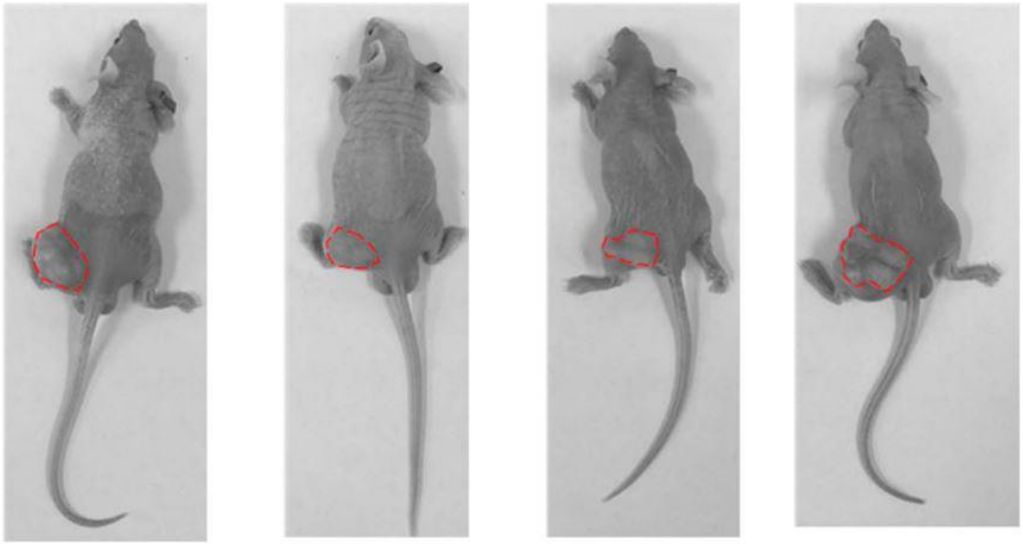


Figure 7: Telomere structure. The telomere is comprised of a repeating 5'-TTAGGG-3' nucleotide motif, stabilized by various proteins. There is a 3' overhang with this motif, and it circles back to self-invade an upstream telomere sequence to form a stable t-loop that effectively caps off both ends of every chromosome.[16]



Left	Right	Left	Right	Left	Right	Left	Right
WTPE	TERT$^{+/-}$	WTPE	TERT$^{+/-}$	WTPE	TERT$^{+/-}$	WTPE	TERT$^{+/-}$
	None		None		None		None

Figure 8: TERT knockout xenograft on mice. xenotransplant of both WT Hela cells (WTPE) and TERT monoallelic knock-out Hela cells (TERT$^{+/-}$) grafted to mouse left and right hind legs, respectively. Table summarizes xenotransplant results; all WTPE grafts developed into tumors, while TERT$^{+/-}$ grafts yielded no tumors.[17]

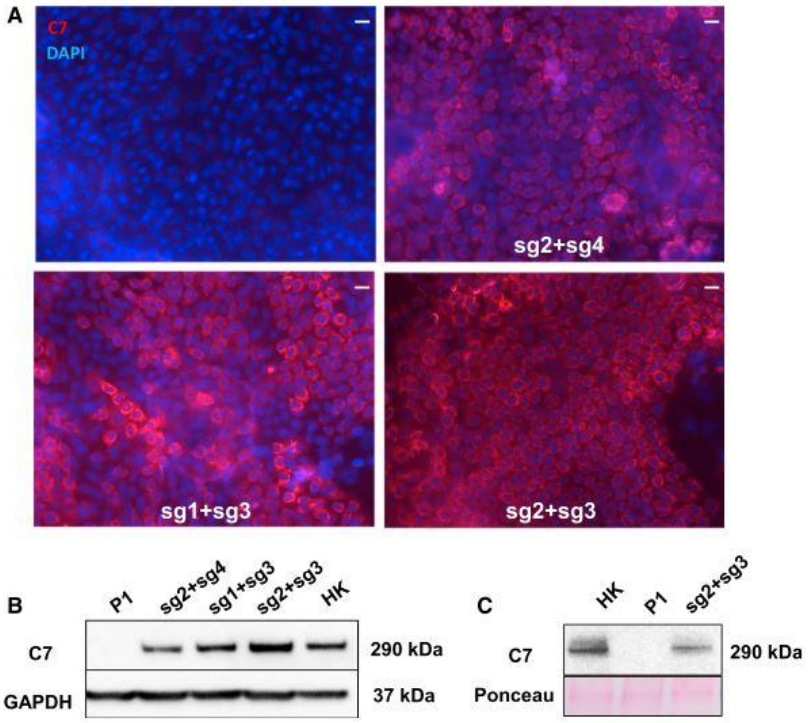


Figure 9: Collagen VII (C7) expression in RDEB keratinocytes. Keratinocytes were either untreated (A, top left), or treated with varying CRISPR/Cas sgRNA combinations to correct C7 gene, and expression was qualified via immunofluorescence. Western blot analyses demonstrating (B) C7 expression in RDEB keratinocytes following CRISPR/Cas treatment with varying combinations of sgRNAs, compared with untreated keratinocytes (P1) and healthy human keratinocytes (HK) and (C) from collected culture supernatant, demonstrating secretion of C7 after CRISPR/Cas-based correction.[21]

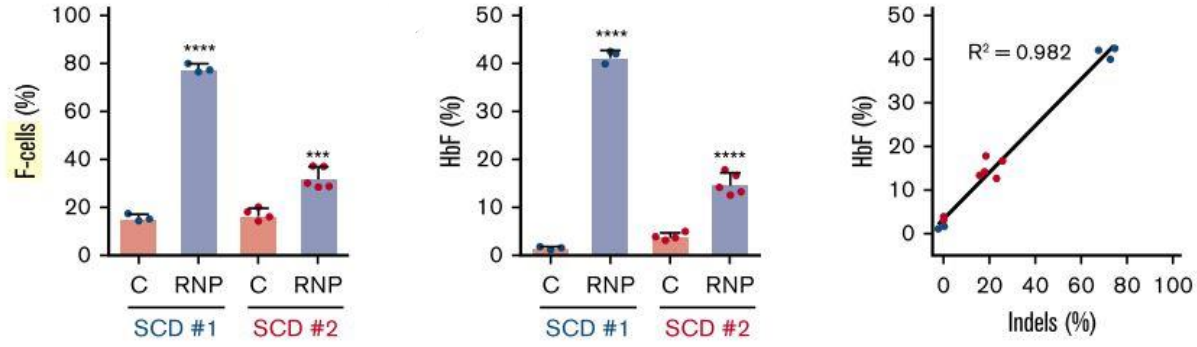


Figure 10: Inducing Fetal Hemoglobin expression. Following transfection with CRISPR/Cas construct targeting a HbF inhibitory TF, cells exhibit (left) substantial increase in HbF expression and (middle) an increase in %HbF in CD235a1 erythroblasts isolated from recipient bone marrow. Additionally, the correlation of the %indels with the %HbF in CD235a1 erythroblasts isolated from recipient bone marrow (right) is illustrated.[23]

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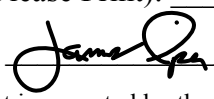
Author(s) of Work(s): James Piper

Depositor's Name (Please Print): James Piper

Author's Signature:  Date: 8/17/2020

If the Deposit Agreement is executed by the Author's Representative, the Representative shall separately execute the Following representation.

I represent that I am authorized by the Author to execute this Deposit Agreement on the behalf of the Author.

Author's Representative Signature: _____ Date: _____