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Genome editing Research Literatures

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Abstract: Genome editing, or genome engineering, or gene editing, is a type of genetic engineering in which DNA is inserted, deleted, modified or replaced in the genome of a living organism. Unlike early genetic engineering techniques that randomly inserts genetic material into a host genome, genome editing targets the insertions to site specific locations. This article introduces recent research reports as references in the related studies.
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Introduction

Genome editing, or genome engineering, or gene editing, is a type of genetic engineering in which DNA is inserted, deleted, modified or replaced in the genome of a living organism. Unlike early genetic engineering techniques that randomly inserts genetic material into a host genome, genome editing targets the insertions to site specific locations. (https://en.wikipedia.org/wiki/Genome_editing). This article introduces recent research reports as references in the related studies.

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History

Genome editing was pioneered in the 1990s,[1] before the advent of the common current nuclease-based gene editing platforms, however, its use was limited by low efficiencies of editing. Genome editing with engineered nucleases, i.e. all three major classes of these enzymes—zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and engineered meganucleases—were selected by *Nature Methods* as the 2011 Method of the Year.[2] The CRISPR-Cas system was selected by *Science* as 2015 Breakthrough of the Year.[3]

As of 2015 four families of engineered nucleases were used: meganucleases, zinc finger nucleases (ZFNs), transcription activator-like effector-based nucleases (TALEN), and the clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) system.[4][5][6][7] Nine genome editors were available as of 2017.[8]

In 2018, the common methods for such editing used engineered nucleases, or "molecular scissors". These nucleases create site-specific double-strand breaks (DSBs) at desired locations in the genome. The induced double-strand breaks are repaired through nonhomologous end-joining (NHEJ) or homologous recombination (HR), resulting in targeted mutations ('edits').

In May 2019, lawyers in China reported, in light of the purported creation by Chinese scientist He Jiankui of the first gene-edited humans (see Lulu and Nana controversy), the drafting of regulations that anyone manipulating the human genome by gene-editing techniques, like CRISPR, would be held responsible for any related adverse consequences.[9] A cautionary perspective on the possible blind spots and risks of CRISPR and related biotechnologies has been recently discussed,[10] focusing on the stochastic nature of cellular control processes.

In February 2020, a US trial safely showed CRISPR gene editing on 3 cancer patients.[11]

Background

Genetic engineering as method of introducing new genetic elements into organisms has been around since the 1970s. One drawback of this technology has been the random nature with which the DNA is inserted into the hosts genome, which can impair or alter other genes within the organism. Although, several methods have been discovered which target the inserted genes to specific sites within an organism genome.[1] It has also enabled the editing of specific sequences within a genome as well as reduced off target effects. This could be used for research purposes, by targeting mutations to specific genes, and in gene therapy. By inserting a functional gene into an organism and targeting it to replace the defective one it could be possible to cure certain genetic diseases.

Gene targeting

Homologous recombination

Early methods to target genes to certain sites within a genome of an organism (called gene targeting) relied on homologous recombination (HR).[12] By creating DNA constructs that contain a template that matches the targeted genome sequence it is possible that the HR processes within the cell will insert the construct at the desired location. Using this method on embryonic stem cells led to the development of transgenic mice with targeted genes knocked out. It has also been possible to knock in genes or alter gene expression patterns.[13] In recognition of their discovery of how homologous recombination can be used to introduce genetic modifications in mice through embryonic stem cells, Mario Capecchi, Martin Evans and Oliver Smithies were awarded the 2007 Nobel Prize for Physiology or Medicine.[14]

Conditional targeting

If a vital gene is knocked out it can prove lethal to the organism. In order to study the function of these genes site specific recombinases (SSR) were used. The two most common types are the Cre-LoxP and Flp-FRT systems. Cre recombinase is an enzyme that removes DNA by homologous recombination between binding sequences known as Lox-P sites. The Flip-FRT system operates in a similar way, with the Flip recombinase recognising FRT sequences. By crossing an organism containing the recombinase sites flanking the gene of interest with an organism that express the SSR under control of tissue specific promoters, it is possible to knock out or switch on genes only in certain cells. These techniques were also used to remove marker genes from transgenic animals. Further modifications of these systems allowed researchers to induce recombination only under certain conditions, allowing genes to be knocked out or expressed at desired times or stages of development.[13]

Process

Double strand break repair

A common form of Genome editing relies on the concept of DNA double stranded break (DSB) repair mechanics. There are two major pathways that repair DSB; non-homologous end joining (NHEJ) and homology directed repair (HDR). NHEJ uses a variety of enzymes to directly join the DNA ends while the more accurate HDR uses a homologous sequence as a template for regeneration of missing DNA sequences at the break point. This can be exploited by creating a vector with the desired genetic elements within a sequence that is homologous to the flanking sequences of a DSB. This will result in the desired change being inserted at the site of the DSB. While HDR based gene editing is similar to the homologous recombination based gene targeting, the rate of recombination is increased by at least three orders of magnitude.[15]

Engineered nucleases

The key to genome editing is creating a DSB at a specific point within the genome. Commonly used restriction enzymes are effective at cutting DNA, but generally recognize and cut at multiple sites. To overcome this challenge and create site-specific DSB, three distinct classes of nucleases have been discovered and bioengineered to date. These are the Zinc finger nucleases (ZFNs), transcription-activator like effector nucleases (TALEN), meganucleases and the clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) system.

Meganucleases

Meganucleases, discovered in the late 1980s, are enzymes in the endonuclease family which are characterized by their capacity to recognize and cut large DNA sequences (from 14 to 40 base pairs).[16] The most widespread and best known meganucleases are the proteins in the LAGLIDADG family, which owe their name to a conserved amino acid sequence.

Meganucleases, found commonly in microbial species, have the unique property of having very long recognition sequences (>14bp) thus making them naturally very specific.[17][18] However, there is virtually no chance of finding the exact meganuclease required to act on a chosen specific DNA sequence. To overcome this challenge, mutagenesis and high throughput screening methods have been used to create meganuclease variants that recognize unique sequences.[18][19] Others have been able to fuse various meganucleases and create hybrid enzymes that recognize a new sequence.[20][21] Yet others have attempted to alter the DNA interacting aminoacids of the meganuclease to design sequence specific meganucelases in a method named rationally designed meganuclease.[22] Another approach involves using computer models to try to predict as accurately as possible the activity of the modified meganucleases and the specificity of the recognized nucleic sequence.[23]

A large bank containing several tens of thousands of protein units has been created. These units can be combined to obtain chimeric meganucleases that recognize the target site, thereby providing research and development tools that meet a wide range of needs (fundamental research, health, agriculture, industry, energy, etc.) These include the industrial-scale production of two meganucleases able to cleave the human XPC gene; mutations in this gene result in *Xeroderma pigmentosum*, a severe monogenic disorder that predisposes the patients to skin cancer and burns whenever their skin is exposed to UV rays.[24]

Meganucleases have the benefit of causing less toxicity in cells than methods such as Zinc finger nuclease (ZFN), likely because of more stringent DNA sequence recognition;[18] however, the construction of sequence-specific enzymes for all possible sequences is

costly and time-consuming, as one is not benefiting from combinatorial possibilities that methods such as ZFNs and TALEN-based fusions utilize.

Zinc finger nucleases

As opposed to meganucleases, the concept behind ZFNs and TALEN technology is based on a non-specific DNA cutting catalytic domain, which can then be linked to specific DNA sequence recognizing peptides such as zinc fingers and transcription activator-like effectors (TALEs).[25] The first step to this was to find an endonuclease whose DNA recognition site and cleaving site were separate from each other, a situation that is not the most common among restriction enzymes.[25] Once this enzyme was found, its cleaving portion could be separated which would be very non-specific as it would have no recognition ability. This portion could then be linked to sequence recognizing peptides that could lead to very high specificity.

Zinc finger motifs occur in several transcription factors. The zinc ion, found in 8% of all human proteins, plays an important role in the organization of their three-dimensional structure. In transcription factors, it is most often located at the protein-DNA interaction sites, where it stabilizes the motif. The C-terminal part of each finger is responsible for the specific recognition of the DNA sequence.

The recognized sequences are short, made up of around 3 base pairs, but by combining 6 to 8 zinc fingers whose recognition sites have been characterized, it is possible to obtain specific proteins for sequences of around 20 base pairs. It is therefore possible to control the expression of a specific gene. It has been demonstrated that this strategy can be used to promote a process of angiogenesis in animals.[26] It is also possible to fuse a protein constructed in this way with the catalytic domain of an endonuclease in order to induce a targeted DNA break, and therefore to use these proteins as genome engineering tools.[27]

The method generally adopted for this involves associating two DNA binding proteins – each containing 3 to 6 specifically chosen zinc fingers – with the catalytic domain of the FokI endonuclease which need to dimerize to cleave the double-strand DNA. The two proteins recognize two DNA sequences that are a few nucleotides apart. Linking the two zinc finger proteins to their respective sequences brings the two FokI domains closer together. FokI requires dimerization to have nuclease activity and this means the specificity increases dramatically as each nuclease partner would recognize a unique DNA sequence. To enhance this effect, FokI nucleases have been engineered that can only function as heterodimers.[28]

Several approaches are used to design specific zinc finger nucleases for the chosen sequences. The most widespread involves combining zinc-finger units with known specificities (modular assembly). Various selection techniques, using bacteria, yeast or mammal cells have been developed to identify the combinations that offer the best specificity and the best cell tolerance. Although the direct genome-wide characterization of zinc finger nuclease activity has not been reported, an assay that measures the total number of double-strand DNA breaks in cells found that only one to two such breaks occur above background in cells treated with zinc finger nucleases with a 24 bp composite recognition site and obligate heterodimer FokI nuclease domains.[28]

The heterodimer functioning nucleases would avoid the possibility of unwanted homodimer activity and thus increase specificity of the DSB. Although the nuclease portions of both ZFNs and TALEN constructs have similar properties, the difference between these engineered nucleases is in their DNA recognition peptide. ZFNs rely on Cys2-His2 zinc fingers and TALEN constructs on TALEs. Both of these DNA recognizing peptide domains have the characteristic that they are naturally found in combinations in their proteins. Cys2-His2 Zinc fingers typically happen in repeats that are 3 bp apart and are found in diverse combinations in a variety of nucleic acid interacting proteins such as transcription factors. Each finger of the Zinc finger domain is completely independent and the binding capacity of one finger is impacted by its neighbor. TALEs on the other hand are found in repeats with a one-to-one recognition ratio between the amino acids and the recognized nucleotide pairs. Because both zinc fingers and TALEs happen in repeated patterns, different combinations can be tried to create a wide variety of sequence specificities.[17] Zinc fingers have been more established in these terms and approaches such as modular assembly (where Zinc fingers correlated with a triplet sequence are attached in a row to cover the required sequence), OPEN (low-stringency selection of peptide domains vs. triplet nucleotides followed by high-stringency selections of peptide combination vs. the final target in bacterial systems), and bacterial one-hybrid screening of zinc finger libraries among other methods have been used to make site specific nucleases.

Zinc finger nucleases are research and development tools that have already been used to modify a range of genomes, in particular by the laboratories in the Zinc Finger Consortium. The US company Sangamo BioSciences uses zinc finger nucleases to carry out research into the genetic engineering of stem cells and the modification of immune cells for therapeutic purposes.[29][30] Modified T lymphocytes are currently undergoing phase I clinical trials to treat a type of brain tumor (glioblastoma) and in the fight against AIDS.[28]

TALEN

Transcription activator-like effector nucleases (TALENs) are specific DNA-binding proteins that feature an array of 33 or 34-amino acid repeats. TALENs are artificial restriction enzymes designed by fusing the DNA cutting domain of a nuclease to TALE domains, which can be tailored to specifically recognize a unique DNA sequence. These fusion proteins serve as readily targetable "DNA scissors" for gene editing applications that enable to perform targeted genome modifications such as sequence insertion, deletion, repair and replacement in living cells.[31] The DNA binding domains, which can be designed to bind any desired DNA sequence, comes from TAL effectors, DNA-binding proteins excreted by plant pathogenic *Xanthomanos app.* TAL effectors consists of repeated domains, each of which contains a highly conserved sequence of 34 amino acids, and recognize a single DNA nucleotide within the target site. The nuclease can create double strand breaks at the target site that can be repaired by error-prone non-homologous end-joining (NHEJ), resulting in gene disruptions through the introduction of small insertions or deletions. Each repeat is conserved, with the exception of the so-called repeat variable di-residues (RVDs) at amino acid positions 12 and 13. The RVDs determine the DNA sequence to which the TALE will bind. This simple one-to-one correspondence between the TALE repeats and the corresponding DNA sequence makes the process of assembling repeat arrays to recognize novel DNA sequences straightforward. These TALEs can be fused to the catalytic domain from a DNA nuclease, FokI, to generate a transcription activator-like effector nuclease (TALEN). The resultant TALEN constructs combine specificity and activity, effectively generating engineered sequence-specific nucleases that bind and cleave DNA sequences only at pre-selected sites. The TALEN target recognition system is based on an easy-to-predict code. TAL nucleases are specific to their target due in part to the length of their 30+ base pairs binding site. TALEN can be performed within a 6 base pairs range of any single nucleotide in the entire genome.[32]

TALEN constructs are used in a similar way to designed zinc finger nucleases, and have three advantages in targeted mutagenesis: (1) DNA binding specificity is higher, (2) off-target effects are lower, and (3) construction of DNA-binding domains is easier.

CRISPR

CRISPRs (Clustered Regularly Interspaced Short Palindromic Repeats) are genetic elements that bacteria use as a kind of acquired immunity to protect against viruses. They consist of short sequences that originate from viral genomes and have been incorporated into the bacterial genome. Cas (CRISPR associated proteins) process these sequences and cut matching viral DNA sequences. By introducing plasmids containing Cas genes and specifically constructed CRISPRs into eukaryotic cells, the eukaryotic genome can be cut at any desired position.[33]

Editing by Nucleobase Modification (BASE Editing)

One of the earliest methods of efficiently editing nucleic acids employs nucleobase modifying enzymes directed by nucleic acid guide sequences was first described in the 1990s and has seen resurgence more recently.[1][34][35][36] This method has the advantage that it does not require breaking the genomic DNA strands, and thus avoids the random insertion and deletions associated with DNA strand breakage. It is only appropriate for precise editing requiring single nucleotide changes and has found to be highly efficient for this type of editing.[36]

Precision and efficiency of engineered nucleases

Meganucleases method of gene editing is the least efficient of the methods mentioned above. Due to the nature of its DNA-binding element and the cleaving element, it is limited to recognizing one potential target every 1,000 nucleotides.[7] ZFN was developed to overcome the limitations of meganuclease. The number of possible targets ZFN can recognized was increased to one in every 140 nucleotides.[7]However, both methods are unpredictable because of their DNA-binding elements affecting each other. As a result, high degrees of expertise and lengthy and costly validations processes are required.

TALE nucleases being the most precise and specific method yields a higher efficiency than the previous two methods. It achieves such efficiency because the DNA-binding element consists of an array of TALE subunits, each of them having the capability of recognizing a specific DNA nucleotide chain independent from others, resulting in a higher number of target sites with high precision. New TALE nucleases take about one week and a few hundred dollars to create, with specific expertise in molecular biology and protein engineering.[7]

CRISPR nucleases have a slightly lower precision when compared to the TALE nucleases. This is caused by the need of having a specific nucleotide at one end in order to produce the guide RNA that CRISPR uses to repair the double-strand break it induces. It has been shown to be the quickest and cheapest method, only costing less than two hundred dollars and a few days of time.[7] CRISPR also requires the least amount of expertise in molecular biology as the design lays in the guide RNA instead of the proteins. One major advantage that CRISPR has over the ZFN and TALEN methods is that it can be directed to target different DNA sequences using its ~80nt CRISPR sgRNAs, while both ZFN and TALEN

methods required construction and testing of the proteins created for targeting each DNA sequence.[37]

Because off-target activity of an active nuclease would have potentially dangerous consequences at the genetic and organismal levels, the precision of meganucleases, ZFNs, CRISPR, and TALEN-based fusions has been an active area of research. While variable figures have been reported, ZFNs tend to have more cytotoxicity than TALEN methods or RNA-guided nucleases, while TALEN and RNA-guided approaches tend to have the greatest efficiency and fewer off-target effects.[38] Based on the maximum theoretical distance between DNA binding and nuclease activity, TALEN approaches result in the greatest precision.[7]

Multiplex Automated Genomic Engineering (MAGE)

The methods for scientists and researchers wanting to study genomic diversity and all possible associated phenotypes were very slow, expensive, and inefficient. Prior to this new revolution, researchers would have to do single-gene manipulations and tweak the genome one little section at a time, observe the phenotype, and start the process over with a different single-gene manipulation.[39] Therefore, researchers at the Wyss Institute at Harvard University designed the MAGE, a powerful technology that improves the process of in vivo genome editing. It allows for quick and efficient manipulations of a genome, all happening in a machine small enough to put on top of a small kitchen table. Those mutations combine with the variation that naturally occurs during cell mitosis creating billions of cellular mutations.

Chemically combined, synthetic single-stranded DNA (ssDNA) and a pool of oligionucleotides are introduced at targeted areas of the cell thereby creating genetic modifications. The cyclical process involves transformation of ssDNA (by electroporation) followed by outgrowth, during which bacteriophage homologous recombination proteins mediate annealing of ssDNAs to their genomic targets. Experiments targeting selective phenotypic markers are screened and identified by plating the cells on differential medias. Each cycle ultimately takes 2.5 hours to process, with additional time required to grow isogenic cultures and characterize mutations. By iteratively introducing libraries of mutagenic ssDNAs targeting multiple sites, MAGE can generate combinatorial genetic diversity in a cell population. There can be up to 50 genome edits, from single nucleotide base pairs to whole genome or gene networks simultaneously with results in a matter of days.[39]

MAGE experiments can be divided into three classes, characterized by varying degrees of scale and complexity: (i) many target sites, single genetic mutations; (ii) single target site, many genetic mutations; and (iii) many target sites, many genetic mutations.[39] An example of class three was reflected in 2009, where Church and colleagues were able to program *Escherichia coli* to produce five times the normal amount of lycopene, an antioxidant normally found in tomato seeds and linked to anti-cancer properties. They applied MAGE to optimize the 1-deoxy-d-xylulose-5-phosphate (DXP) metabolic pathway in Escherichia coli to overproduce isoprenoid lycopene. It took them about 3 days and just over $1,000 in materials. The ease, speed, and cost efficiency in which MAGE can alter genomes can transform how industries approach the manufacturing and production of important compounds in the bioengineering, bioenergy, biomedical engineering, synthetic biology, pharmaceutical, agricultural, and chemical industries.

Applications

As of 2012 efficient genome editing had been developed for a wide range of experimental systems ranging from plants to animals, often beyond clinical interest, and was becoming a standard experimental strategy in research labs.[40] The recent generation of rat, zebrafish, maize and tobacco ZFN-mediated mutants and the improvements in TALEN-based approaches testify to the significance of the methods, and the list is expanding rapidly. Genome editing with engineered nucleases will likely contribute to many fields of life sciences from studying gene functions in plants and animals to gene therapy in humans. For instance, the field of synthetic biology which aims to engineer cells and organisms to perform novel functions, is likely to benefit from the ability of engineered nuclease to add or remove genomic elements and therefore create complex systems.[40] In addition, gene functions can be studied using stem cells with engineered nucleases.

Listed below are some specific tasks this method can carry out:

- Targeted gene mutation
- Gene therapy
- Creating chromosome rearrangement
- Study gene function with stem cells
- Transgenic animals
- Endogenous gene labeling
- Targeted transgene addition

Targeted gene modification in animals

The combination of recent discoveries in genetic engineering, particularly gene editing and the latest improvement in bovine reproduction technologies (e.g. *in vitro* embryo culture) allows for genome editing directly in fertilised oocytes using synthetic highly specific endonucleases. RNA-guided endonucleases:clustered regularly interspaced short palindromic repeats associated Cas9 (CRISPR/Cas9)

are a new tool, further increasing the range of methods available**.** In particular CRISPR/Cas9 engineered endonucleases allows the use of multiple guide RNAs for simultaneous Knockouts (KO) in one step by cytoplasmic direct injection (CDI) on mammalian zygotes.[41]

Furthermore, gene editing can be applied to certain types of fish in aquaculture such as Atlantic salmon. Gene editing in fish is currently experimental, but the possibilities include growth, disease resistance, sterility, controlled reproduction, and colour. Selecting for these traits can allow for a more sustainable environment and better welfare for the fish.[42]

Aqu Advantage salmon is a genetically modified Atlantic salmon developed by AquaBounty Technologies. The growth hormone-regulating gene in the Atlantic salmon is replaced with the growth hormone-regulating gene from the Pacific Chinook salmon and a promoter sequence from the ocean pout[43].

Thanks to the parallel development of single-cell transcriptomics, genome editing and new stem cell models we are now entering a scientifically exciting period where functional genetics is no longer restricted to animal models but can be performed directly in human samples. Single-cell gene expression analysis has resolved a transcriptional road-map of human development from which key candidate genes are being identified for functional studies. Using global transcriptomics data to guide experimentation, the CRISPR based genome editing tool has made it feasible to disrupt or remove key genes in order to elucidate function in a human setting.[44]

Targeted gene modification in plants

Genome editing using Meganuclease,[45] ZFNs, and TALEN provides a new strategy for genetic manipulation in plants and are likely to assist in the engineering of desired plant traits by modifying endogenous genes. For instance, site-specific gene addition in major crop species can be used for 'trait stacking' whereby several desired traits are physically linked to ensure their co-segregation during the breeding processes.[28] Progress in such cases have been recently reported in *Arabidopsis thaliana*[46][47][48] and *Zea mays*. In *Arabidopsis thaliana*, using ZFN-assisted gene targeting, two herbicide-resistant genes (tobacco acetolactate synthase SuRA and SuRB) were introduced to SuR loci with as high as 2% transformed cells with mutations.[46] In Zea mays, disruption of the target locus was achieved by ZFN-induced DSBs and the resulting NHEJ. ZFN was also used to drive herbicide-tolerance gene expression cassette (PAT) into the targeted endogenous locus IPK1 in this case.[49] Such genome modification observed in the regenerated plants has been shown to be inheritable and was transmitted to the next generation.[49] A potentially successful example of the application of genome editing techniques in crop improvement can be found in banana, where scientists used CRISPR/Cas9 editing to inactivate the endogenous banana streak virus in the B genome of banana (*Musa* spp.) to overcome a major challenge in banana breeding.[50]

In addition, TALEN-based genome engineering has been extensively tested and optimized for use in plants.[51] TALEN fusions have also been used by a U.S. food ingredient company, Calyxt,[52] to improve the quality of soybean oil products[53] and to increase the storage potential of potatoes[54]

Several optimizations need to be made in order to improve editing plant genomes using ZFN-mediated targeting.[55] There is a need for reliable design and subsequent test of the nucleases, the absence of toxicity of the nucleases, the appropriate choice of the plant tissue for targeting, the routes of induction of enzyme activity, the lack of off-target mutagenesis, and a reliable detection of mutated cases.[55]

A common delivery method for CRISPR/Cas9 in plants is *Agrobacterium*-based transformation.[56] T-DNA is introduced directly into the plant genome by a T4SS mechanism. Cas9 and gRNA-based expression cassettes are turned into Ti plasmids, which are transformed in *Agrobacterium* for plant application.[56] To improve Cas9 delivery in live plants, viruses are being used more effective transgene delivery.[56]

Research

Gene therapy

The ideal gene therapy practice is that which replaces the defective gene with a normal allele at its natural location. This is advantageous over a virally delivered gene as there is no need to include the full coding sequences and regulatory sequences when only a small proportions of the gene needs to be altered as is often the case.[57] The expression of the partially replaced genes is also more consistent with normal cell biology than full genes that are carried by viral vectors.

The first clinical use of TALEN-based genome editing was in the treatment of CD19+ acute lymphoblastic leukemia in an 11-month old child in 2015. Modified donor T cells were engineered to attack the leukemia cells, to be resistant to Alemtuzumab, and to evade detection by the host immune system after introduction.[58][59]

Extensive research has been done in cells and animals using CRISPR-Cas9 to attempt to correct genetic mutations which cause genetic diseases such as Down syndrome, spina bifida, anencephaly, and Turner and Klinefelter syndromes.[60]

In February 2019, medical scientists working with Sangamo Therapeutics, headquartered in Richmond, California, announced the first ever "in

body" human gene editing therapy to permanently alter DNA - in a patient with Hunter Syndrome.[61] Clinical trials by Sangamo involving gene editing using Zinc Finger Nuclease (ZFN) are ongoing.[62]

Eradicating diseases

Researchers have used CRISPR-Cas9 gene drives to modify genes associated with sterility in *A. gambiae*, the vector for malaria.[63] This technique has further implications in eradicating other vector borne diseases such as yellow fever, dengue, and Zika.[64]

The CRISPR-Cas9 system can be programmed to modulate the population of any bacterial species by targeting clinical genotypes or epidemiological isolates. It can selectively enable the beneficial bacterial species over the harmful ones by eliminating pathogen, which gives it an advantage over broad-spectrum antibiotics.[39]

Antiviral applications for therapies targeting human viruses such as HIV, herpes, and hepatitis B virus are under research. CRISPR can be used to target the virus or the host to disrupt genes encoding the virus cell-surface receptor proteins.[37] In November 2018, He Jiankui announced that he had edited two human embryos, to attempt to disable the gene for CCR5, which codes for a receptor that HIV uses to enter cells. He said that twin girls, Lulu and Nana, had been born a few weeks earlier. He said that the girls still carried functional copies of CCR5 along with disabled CCR5 (mosaicism) and were still vulnerable to HIV. The work was widely condemned as unethical, dangerous, and premature.[65]

In January 2019, scientists in China reported the creation of five identical cloned gene-edited monkeys, using the same cloning technique that was used with Zhong Zhong and Hua Hua – the first ever cloned monkeys - and Dolly the sheep, and the same gene-editing Crispr-Cas9 technique allegedly used by He Jiankui in creating the first ever gene-modified human babies Lulu and Nana. The monkey clones were made in order to study several medical diseases.[66][67]

In the near future the new CRISPR system will also be able to eradicate diseases and conditions that humans are predisposed for. With this new technology scientists will be able to take the genes of a human sperm cell and egg, and replace the genes that activate cancer or other abnormal or unwanted defects. This will take the stress off from parents worrying about having a child and not able to live a normal life. After just one generation of this process, the entire future of the human race would never have to worry about the problems of deformities or predisposed conditions.[68]

Prospects and limitations

In the future, an important goal of research into genome editing with engineered nucleases must be the improvement of the safety and specificity of the nucleases action.[69] For example, improving the ability to detect off-target events can improve our ability to learn about ways of preventing them. In addition, zinc-fingers used in ZFNs are seldom completely specific, and some may cause a toxic reaction. However, the toxicity has been reported to be reduced by modifications done on the cleavage domain of the ZFN.[57]

In addition, research by Dana Carroll into modifying the genome with engineered nucleases has shown the need for better understanding of the basic recombination and repair machinery of DNA. In the future, a possible method to identify secondary targets would be to capture broken ends from cells expressing the ZFNs and to sequence the flanking DNA using high-throughput sequencing.[57]

Because of the ease of use and cost-efficiency of CRISPR, extensive research is currently being done on it. There are now more publications on CRISPR than ZFN and TALEN despite how recent the discovery of CRISPR is.[37] Both CRISPR and TALEN are favored to be the choices to be implemented in large-scale productions due to their precision and efficiency.

Genome editing occurs also as a natural process without artificial genetic engineering. The agents that are competent to edit genetic codes are viruses or subviral RNA-agents.

Although GEEN has higher efficiency than many other methods in reverse genetics, it is still not highly efficient; in many cases less than half of the treated populations obtain the desired changes.[46] For example, when one is planning to use the cell's NHEJ to create a mutation, the cell's HDR systems will also be at work correcting the DSB with lower mutational rates.

Traditionally, mice have been the most common choice for researchers as a host of a disease model. CRISPR can help bridge the gap between this model and human clinical trials by creating transgenic disease models in larger animals such as pigs, dogs, and non-human primates.[70][71] Using the CRISPR-Cas9 system, the programmed Cas9 protein and the sgRNA can be directly introduced into fertilized zygotes to achieve the desired gene modifications when creating transgenic models in rodents. This allows bypassing of the usual cell targeting stage in generating transgenic lines, and as a result, it reduces generation time by 90%.[71]

One potential that CRISPR brings with its effectiveness is the application of xenotransplantation. In previous research trials, CRISPR demonstrated the ability to target and eliminate endogenous retroviruses, which reduces the risk of transmitting diseases and reduces immune barriers.[37] Eliminating these

problems improves donor organ function, which brings this application closer to a reality.

In plants, genome editing is seen as a viable solution to the conservation of biodiversity. Gene drive are a potential tool to alter the reproductive rate of invasive species, although there are significant associated risks.[72]

Human enhancement

Many transhumanists see genome editing as a potential tool for human enhancement.[73][74][75] Australian biologist and Professor of Genetics David Andrew Sinclair notes that "the new technologies with genome editing will allow it to be used on individuals (...) to have (...) healthier children" – designer babies.[76] According to a September 2016 report by the Nuffield Council on Bioethics in the future it may be possible to enhance people with genes from other organisms or wholly synthetic genes to for example improve night vision and sense of smell.[77][78]

The American National Academy of Sciences and National Academy of Medicine issued a report in February 2017 giving qualified support to human genome editing.[79] They recommended that clinical trials for genome editing might one day be permitted once answers have been found to safety and efficiency problems "but only for serious conditions under stringent oversight."[80]

Risks

In the 2016 *Worldwide Threat Assessment of the US Intelligence Community* statement United States Director of National Intelligence, James R. Clapper, named genome editing as a potential weapon of mass destruction, stating that genome editing conducted by countries with regulatory or ethical standards "different from Western countries" probably increases the risk of the creation of harmful biological agents or products. According to the statement the broad distribution, low cost, and accelerated pace of development of this technology, its deliberate or unintentional misuse might lead to far-reaching economic and national security implications.[81][82][83] For instance technologies such as CRISPR could be used to make "killer mosquitoes" that cause plagues that wipe out staple crops.[83]

According to a September 2016 report by the Nuffield Council on Bioethics, the simplicity and low cost of tools to edit the genetic code will allow amateurs – or "biohackers" – to perform their own experiments, posing a potential risk from the release of genetically modified bugs. The review also found that the risks and benefits of modifying a person's genome – and having those changes pass on to future generations – are so complex that they demand urgent ethical scrutiny. Such modifications might have unintended consequences which could harm not only the child, but also their future children, as the altered gene would be in their sperm or eggs.[77][78] In 2001 Australian researchers Ronald Jackson and Ian Ramshaw were criticized for publishing a paper in the Journal of Virology that explored the potential control of mice, a major pest in Australia, by infecting them with an altered mousepox virus that would cause infertility as the provided sensitive information could lead to the manufacture of biological weapons by potential bioterrorists who might use the knowledge to create vaccine resistant strains of other pox viruses, such as smallpox, that could affect humans.[78][84] Furthermore, there are additional concerns about the ecological risks of releasing gene drives into wild populations.[78][85][86]

PubMed Literatures

Amritha, P. P. and J. M. Shah (2021). "Can genetic engineering-based methods for gene function identification be eclipsed by genome editing in plants? A comparison of methodologies." Mol Genet Genomics.

Finding and explaining the functions of genes in plants have promising applications in crop improvement and bioprospecting and hence, it is important to compare various techniques available for gene function identification in plants. Today, the most popular technology among researchers to identify the functions of genes is the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9)-based genome editing method. But by no means can we say that CRISPR/Cas9 is the go-to method for all purposes. It comes with its own baggage. Researchers will agree and have lived through at least seven more technologies deployed to find the functions of genes, which come under three umbrellas: 1. genetic engineering, 2. transient expression, and 3. chemical/physical mutagenesis. Each of the methods evolved when the previous one ran into an insurmountable problem. In this review, we compare the eight technologies against one another on 14 parameters. This review lays bare the pros and cons, and similarities and dissimilarities of various methods. Every method comes with its advantages and disadvantages. For example, the CRISPR/Cas9-based genome editing is an excellent method for modifying gene sequences, creating allelic versions of genes, thereby aiding the understanding of gene function. But it comes with the baggage of unwanted or off-target mutations. Then, we have methods based on random or targeted knockout of the gene, knockdown, and overexpression of the gene. Targeted disruption of genes is required for complete knockout of gene function, which may not be accomplished by editing. We have also discussed the strategies to overcome the shortcomings of the targeted gene-knockout and the CRISPR/Cas9-based methods. This review serves as a

comprehensive guide towards the understanding and comparison of various technologies available for gene function identification in plants and hence, it will find application for crop improvement and bioprospecting related research.

Annas, G. J. (2020). "Genome Editing 2020: Ethics and Human Rights in Germline Editing in Humans and Gene Drives in Mosquitoes." Am J Law Med **46**(2-3): 143-165.

The moon landing, now more than a half century in the past, has turned out to be the culmination of human space travel, rather than its beginning. Genetic engineering, especially applications of CRISPR, now presents the most publicly-discussed engineering challenges-and not just technical, but ethical as well. In this article, I will use the two most controversial genomic engineering applications to help identify the ethics and human rights implications of these research projects. Each of these techniques directly modifies the mechanisms of evolution, threatens to alter our views of ourselves as humans and our planet as our home, and presents novel informed consent and dual use challenges: human genome editing and gene drives in insects.I begin with a discussion of so far disastrously unsuccessful attempts to regulate germline editing in humans, including a summary of the first application of germline genome editing in humans and its aftermath. I then turn to a discussion of setting ethical standards for a genomic technology that has not yet been deployed in nature-gene drives. Finally, I end by suggesting that human rights can and should be directly applicable to defining the ethics of genomic research.

Ansai, S., et al. (2021). "Genome editing reveals fitness effects of a gene for sexual dichromatism in Sulawesian fishes." Nat Commun **12**(1): 1350.

Sexual selection drives rapid phenotypic diversification of mating traits. However, we know little about the causative genes underlying divergence in sexually selected traits. Here, we investigate the genetic basis of male mating trait diversification in the medaka fishes (genus Oryzias) from Sulawesi, Indonesia. Using linkage mapping, transcriptome analysis, and genome editing, we identify csf1 as a causative gene for red pectoral fins that are unique to male Oryzias woworae. A cis-regulatory mutation enables androgen-induced expression of csf1 in male fins. csf1-knockout males have reduced red coloration and require longer for mating, suggesting that coloration can contribute to male reproductive success. Contrary to expectations, non-red males are more attractive to a predatory fish than are red males. Our results demonstrate that integrating genomics with genome editing enables us to identify causative genes underlying sexually selected traits and provides a new avenue for testing theories of sexual selection.

Badia, R., et al. (2017). "Antiviral treatment strategies based on gene silencing and genome editing." Curr Opin Virol **24**: 46-54.

The ability of some viruses to establish latently infected chronic reservoirs that escape to immune control becomes a major roadblock that impedes the cure of these infections. Therefore, new alternatives are needed to pursuit the eradication of viral persistent infections. Gene silencing technologies are in constant evolution and provide an outstanding sequence specificity that allows targeting any coding sequence of interest. Here we provide an overview of the development of gene silencing technologies ranging from initially RNA interference to the recently developed CRISPR/Cas9 and their potential as new antiviral strategies focusing on the eradication of HIV.

Bae, S. J., et al. (2020). "Multiplex Gene Disruption by Targeted Base Editing of Yarrowia lipolytica Genome Using Cytidine Deaminase Combined with the CRISPR/Cas9 System." Biotechnol J **15**(1): e1900238.

The oleaginous yeast Yarrowia lipolytica has a tendency to use the non-homologous end joining repair (NHEJ) over the homology directed recombination as double-strand breaks (DSB) repair system, making it difficult to edit the genome using homologous recombination. A recently developed Target-AID (activation-induced cytidine deaminase) base editor, designed to recruit cytidine deaminase (CDA) to the target DNA locus via the CRISPR/Cas9 system, can directly induce C to T mutation without DSB and donor DNA. In this study, this system is adopted in Y. lipolytica for multiplex gene disruption. Target-specific gRNA(s) and a fusion protein consisting of a nickase Cas9, pmCDA1, and uracil DNA glycosylase inhibitor are expressed from a single plasmid to disrupt target genes by introducing a stop codon via C to T mutation within the mutational window. Deletion of the KU70 gene involved in the NHEJ prevents the generation of indels by base excision repair following cytidine deamination, increasing the accuracy of genome editing. Using this Target-AID system with optimized expression levels of the base editor, single gene disruption and simultaneous double gene disruption are achieved with the efficiencies up to 94% and 31%, respectively, demonstrating this base editing system as a convenient genome editing tool in Y. lipolytica.

Banakar, R., et al. (2020). "Comparison of CRISPR-Cas9/Cas12a Ribonucleoprotein Complexes for Genome Editing Efficiency in the Rice Phytoene Desaturase (OsPDS) Gene." Rice (N Y) **13**(1): 4.

BACKGROUND: Delivery of CRISPR reagents into cells as ribonucleoprotein (RNP) complexes enables transient editing, and avoids CRISPR reagent integration in the genomes. Another technical advantage is that RNP delivery can bypass the need of cloning and vector construction steps. In this work we compared efficacies and types of edits for three Cas9 (WT Cas9 nuclease, HiFi Cas9 nuclease, Cas9 D10A nickase) and two Cas12a nucleases (AsCas12a and LbCas12a), using the rice phytoene desaturase (PDS) gene as a target site. FINDINGS: Delivery of two Cas9 nucleases (WT Cas9, and HiFi Cas9) and one Cas12a nuclease (LbCas12a) resulted in targeted mutagenesis of the PDS gene. LbCas12a had a higher editing efficiency than that of WT Cas9 and HiFi Cas9. Editing by Cas9 enzymes resulted in indels (1-2 bp) or larger deletions between 20-bp to 30-bp, which included the loss of the PAM site; whereas LbCas12a editing resulted in deletions ranging between 2 bp to 20 bp without the loss of the PAM site. CONCLUSIONS: In this work, when a single target site of the rice gene OsPDS was evaluated, the LbCas12a RNP complex achieved a higher targeted mutagenesis frequency than the AsCas12a or Cas9 RNPs.

Benakanakere, M. R., et al. (2016). "Investigation of the functional role of human Interleukin-8 gene haplotypes by CRISPR/Cas9 mediated genome editing." Sci Rep **6**: 31180.

Interleukin-8 (IL-8) gene polymorphisms have been considered as susceptibility factors in periodontal disease. However, the functional roles of IL-8 gene haplotypes have not been investigated. Here, we demonstrate for the first time the use of the CRISPR/Cas9 system to engineer the IL-8 gene, and tested the functionality of different haplotypes. Two sgRNAs vectors targeting the IL-8 gene and the naked homologous repair DNA carrying different haplotypes were used to successfully generate HEK293T cells carrying the AT genotype at the first SNP - rs4073 (alias -251), TT genotype at the second SNP - rs2227307 (alias +396), TC or CC genotypes at the third SNP - rs2227306 (alias +781) at the IL-8 locus. When stimulated with Poly I:C, ATC/TTC haplotype, cells significantly up-regulated the IL-8 at both transcriptional and translational levels. To test whether ATC/TTC haplotype is functional, we used a trans-well assay to measure the transmigration of primary neutrophils incubated with supernatants from the Poly I:C stimulation experiment. ATC/TTC haplotype cells significantly increased transmigration of neutrophils confirming the functional role for this IL-8 haplotype. Taken together, our data provides evidence that carriage of the ATC/TTC haplotype in itself may increase the influx of neutrophils in inflammatory lesions and influence disease susceptibility.

Bennett, E. P., et al. (2020). "INDEL detection, the 'Achilles heel' of precise genome editing: a survey of methods for accurate profiling of gene editing induced indels." Nucleic Acids Res **48**(21): 11958-11981.

Advances in genome editing technologies have enabled manipulation of genomes at the single base level. These technologies are based on programmable nucleases (PNs) that include meganucleases, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated 9 (Cas9) nucleases and have given researchers the ability to delete, insert or replace genomic DNA in cells, tissues and whole organisms. The great flexibility in re-designing the genomic target specificity of PNs has vastly expanded the scope of gene editing applications in life science, and shows great promise for development of the next generation gene therapies. PN technologies share the principle of inducing a DNA double-strand break (DSB) at a user-specified site in the genome, followed by cellular repair of the induced DSB. PN-elicited DSBs are mainly repaired by the non-homologous end joining (NHEJ) and the microhomology-mediated end joining (MMEJ) pathways, which can elicit a variety of small insertion or deletion (indel) mutations. If indels are elicited in a protein coding sequence and shift the reading frame, targeted gene knock out (KO) can readily be achieved using either of the available PNs. Despite the ease by which gene inactivation in principle can be achieved, in practice, successful KO is not only determined by the efficiency of NHEJ and MMEJ repair; it also depends on the design and properties of the PN utilized, delivery format chosen, the preferred indel repair outcomes at the targeted site, the chromatin state of the target site and the relative activities of the repair pathways in the edited cells. These variables preclude accurate prediction of the nature and frequency of PN induced indels. A key step of any gene KO experiment therefore becomes the detection, characterization and quantification of the indel(s) induced at the targeted genomic site in cells, tissues or whole organisms. In this survey, we briefly review naturally occurring indels and their detection. Next, we review the methods that have been developed for detection of PN-induced indels. We briefly outline the experimental steps and describe the pros and cons of the various methods to help users decide a suitable method for their editing application. We highlight recent advances that enable accurate and sensitive quantification of indel events in cells regardless of their genome complexity, turning a complex pool of different indel events into informative

indel profiles. Finally, we review what has been learned about PN-elicited indel formation through the use of the new methods and how this insight is helping to further advance the genome editing field.

Blighe, K., et al. (2018). "Gene editing in the context of an increasingly complex genome." BMC Genomics **19**(1): 595.

The reporting of the first draft of the human genome in 2000 brought with it much hope for the future in what was felt as a paradigm shift toward improved health outcomes. Indeed, we have now mapped the majority of variation across human populations with landmark projects such as 1000 Genomes; in cancer, we have catalogued mutations across the primary carcinomas; whilst, for other diseases, we have identified the genetic variants with strongest association. Despite this, we are still awaiting the genetic revolution in healthcare to materialise and translate itself into the health benefits for which we had hoped. A major problem we face relates to our underestimation of the complexity of the genome, and that of biological mechanisms, generally. Fixation on DNA sequence alone and a 'rigid' mode of thinking about the genome has meant that the folding and structure of the DNA molecule -and how these relate to regulation- have been underappreciated. Projects like ENCODE have additionally taught us that regulation at the level of RNA is just as important as that at the spatiotemporal level of chromatin.In this review, we chart the course of the major advances in the biomedical sciences in the era pre- and post the release of the first draft sequence of the human genome, taking a focus on technology and how its development has influenced these. We additionally focus on gene editing via CRISPR/Cas9 as a key technique, in particular its use in the context of complex biological mechanisms. Our aim is to shift the mode of thinking about the genome to that which encompasses a greater appreciation of the folding of the DNA molecule, DNA- RNA/protein interactions, and how these regulate expression and elaborate disease mechanisms.Through the composition of our work, we recognise that technological improvement is conducive to a greater understanding of biological processes and life within the cell. We believe we now have the technology at our disposal that permits a better understanding of disease mechanisms, achievable through integrative data analyses. Finally, only with greater understanding of disease mechanisms can techniques such as gene editing be faithfully conducted.

Bothmer, A., et al. (2020). "Detection and Modulation of DNA Translocations During Multi-Gene Genome Editing in T Cells." CRISPR J **3**(3): 177-187.

Multiplexed genome editing with DNA endonucleases has broad application, including for cellular therapies, but chromosomal translocations, natural byproducts of inducing simultaneous genomic breaks, have not been explored in detail. Here we apply various CRISPR-Cas nucleases to edit the T cell receptor alpha and beta 2 microglobulin genes in human primary T cells and comprehensively evaluate the frequency and stability of the resulting translocations. A thorough translocation frequency analysis using three orthogonal methods (droplet digital PCR, unidirectional sequencing, and metaphase fluorescence in situ hybridization) yielded comparable results and an overall translocation rate of approximately 7% between two simultaneous CRISPR-Cas9 induced edits. In addition, we show that chromosomal translocations can be reduced when using different nuclease combinations, or by the presence of a homologous single stranded oligo donor for multiplexed genome editing. Importantly, the two different approaches for translocation reduction are compatible with cell therapy applications.

Boulad, F., et al. (2018). "Gene Therapy and Genome Editing." Hematol Oncol Clin North Am **32**(2): 329-342.

The beta-thalassemias are inherited blood disorders that result from insufficient production of the beta-chain of hemoglobin. More than 200 different mutations have been identified. beta-Thalassemia major requires life-long transfusions. The only cure for severe beta-thalassemia is to provide patients with hematopoietic stem cells. Globin gene therapy promises a curative autologous stem cell transplantation without the immunologic complications of allogeneic transplantation. The future directions of gene therapy include enhancement of lentiviral vector-based approaches, fine tuning of the conditioning regimen, and the design of safer vectors. Progress in genetic engineering bodes well for finding a cure for severe globin disorders.

Brown, A. J., et al. (2013). "Whole-rat conditional gene knockout via genome editing." Nat Methods **10**(7): 638-640.

Animal models with genetic modifications under temporal and/or spatial control are invaluable to functional genomics and medical research. Here we report the generation of tissue-specific knockout rats via microinjection of zinc-finger nucleases (ZFNs) into fertilized eggs. We generated rats with loxP-flanked (floxed) alleles and a tyrosine hydroxylase promoter-driven cre allele and demonstrated Cre-dependent gene disruption in vivo. Pronuclear microinjection of ZFNs, shown by our data to be an efficient and rapid method

for creating conditional knockout rats, should also be applicable in other species.

Bryant, J. M., et al. (2017). "CRISPR/Cas9 Genome Editing Reveals That the Intron Is Not Essential for var2csa Gene Activation or Silencing in Plasmodium falciparum." mBio **8**(4).

Plasmodium falciparum relies on monoallelic expression of 1 of 60 var virulence genes for antigenic variation and host immune evasion. Each var gene contains a conserved intron which has been implicated in previous studies in both activation and repression of transcription via several epigenetic mechanisms, including interaction with the var promoter, production of long noncoding RNAs (lncRNAs), and localization to repressive perinuclear sites. However, functional studies have relied primarily on artificial expression constructs. Using the recently developed P. falciparum clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system, we directly deleted the var2csa P. falciparum 3D7_1200600 (Pf3D7_1200600) endogenous intron, resulting in an intronless var gene in a natural, marker-free chromosomal context. Deletion of the var2csa intron resulted in an upregulation of transcription of the var2csa gene in ring-stage parasites and subsequent expression of the PfEMP1 protein in late-stage parasites. Intron deletion did not affect the normal temporal regulation and subsequent transcriptional silencing of the var gene in trophozoites but did result in increased rates of var gene switching in some mutant clones. Transcriptional repression of the intronless var2csa gene could be achieved via long-term culture or panning with the CD36 receptor, after which reactivation was possible with chondroitin sulfate A (CSA) panning. These data suggest that the var2csa intron is not required for silencing or activation in ring-stage parasites but point to a subtle role in regulation of switching within the var gene family.IMPORTANCEPlasmodium falciparum is the most virulent species of malaria parasite, causing high rates of morbidity and mortality in those infected. Chronic infection depends on an immune evasion mechanism termed antigenic variation, which in turn relies on monoallelic expression of 1 of ~60 var genes. Understanding antigenic variation and the transcriptional regulation of monoallelic expression is important for developing drugs and/or vaccines. The var gene family encodes the antigenic surface proteins that decorate infected erythrocytes. Until recently, studying the underlying genetic elements that regulate monoallelic expression in P. falciparum was difficult, and most studies relied on artificial systems such as episomal reporter genes. Our study was the first to use CRISPR/Cas9 genome editing for the functional study of an important, conserved genetic element of var genes-the intron-in an endogenous, episome-free manner. Our findings shed light on the role of the var gene intron in transcriptional regulation of monoallelic expression.

Burle-Caldas, G. A., et al. (2018). "Assessment of two CRISPR-Cas9 genome editing protocols for rapid generation of Trypanosoma cruzi gene knockout mutants." Int J Parasitol **48**(8): 591-596.

CRISPR/Cas9 technology has been used to edit genomes in a variety of organisms. Using the GP72 gene as a target sequence, we tested two distinct approaches to generate Trypanosoma cruzi knockout mutants using the Cas9 nuclease and in vitro transcribed single guide RNA. Highly efficient rates of disruption of GP72 were achieved either by transfecting parasites stably expressing Streptococcus pyogenes Cas9 with single guide RNA or by transfecting wild type parasites with recombinant Staphylococcus aureus Cas9 previously associated with single guide RNA. In both protocols, we used single-stranded oligonucleotides as a repair template for homologous recombination and insertion of stop codons in the target gene.

Cai, M. and Y. Yang (2014). "Targeted genome editing tools for disease modeling and gene therapy." Curr Gene Ther **14**(1): 2-9.

The development of custom-designed nucleases (CDNs), including zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), has made it possible to perform precise genetic engineering in many cell types and species. More recently, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system has been successfully employed for genome editing. These RNA-guided DNA endonucleases are shown to be more efficient and flexible than CDNs and hold great potential for applications in both biological studies and medicine. Here, we review the progress that has been made for all three genome editing technologies in modifying both cells and model organisms, compare important aspects of each approach, and summarize the applications of these tools in disease modeling and gene therapy. In the end, we discuss future prospects of the field.

Canoy, R. J., et al. (2020). "Easy and robust electrotransfection protocol for efficient ectopic gene expression and genome editing in human B cells." Gene Ther.

B-cell lines and primary PBMCs are notoriously hard to transfect, thus making genome editing, ectopic gene expression, or gene silencing experiments particularly tedious. Here we propose a novel efficient and reproducible protocol for electrotransfection of lymphoblastoid, B-cell

lymphoma, leukemia cell lines, and B cells from PBMCs. The proposed protocol requires neither costly equipment nor expensive reagents; it can be used with small or large plasmids. Transfection and viability rates of about 79% and 58%, respectively, have been routinely achieved by optimizing the salt concentration in the electrotransfection medium and the amount of plasmid used. A validation of the protocol was obtained via the generation of a TP53(-/)(-) RPMI8866 lymphoblastoid cell line which should prove useful in future hematological and blood cancer studies.

Cao, M., et al. (2018). "CRISPR-Mediated Genome Editing and Gene Repression in Scheffersomyces stipitis." Biotechnol J **13**(9): e1700598.

Scheffersomyces stipitis, renowned for its native xylose-utilizing capacity, has recently demonstrated its potential in producing health-promoting shikimate pathway derivatives. However, its broader application is hampered by the low transformation efficiency and the lack of genetic engineering tools to enable sophisticated genomic manipulations. S. stipitis employs the predominant non-homologous end joining (NHEJ) mechanism for repairing DNA double-strand breaks (DSB), which is less desired due to its incompetence in achieving precise genome editing. Using CRISPR technology, here a ku70Deltaku80Delta deficient strain in which homologous recombination (HR)-based genome editing appeared dominant for the first time in S. stipitis is constructed. To build all essential tools for efficiently manipulating this highly promising nonconventional microbial host, the gene knockdown tool is also established, and repression efficiency is improved by incorporating a transcriptional repressor Mxi1 into the CRISPR-dCas9 platform. All these results are obtained with the improved transformation efficiency, which is 191-fold higher than that obtained with the traditional parameters used in yeast transformation. This work paves the way for advancing a new microbial chassis and provides a guideline for developing efficient CRISPR tools in other nonconventional yeasts.

Capriotti, L., et al. (2020). "Biotechnological Approaches: Gene Overexpression, Gene Silencing, and Genome Editing to Control Fungal and Oomycete Diseases in Grapevine." Int J Mol Sci **21**(16).

Downy mildew, powdery mildew, and grey mold are some of the phytopathological diseases causing economic losses in agricultural crops, including grapevine, worldwide. In the current scenario of increasing global warming, in which the massive use of agrochemicals should be limited, the management of fungal disease has become a challenge. The knowledge acquired on candidate resistant (R) genes having an active role in plant defense mechanisms has allowed numerous breeding programs to integrate these traits into selected cultivars, even though with some limits in the conservation of the proper qualitative characteristics of the original clones. Given their gene-specific mode of action, biotechnological techniques come to the aid of breeders, allowing them to generate simple and fast modifications in the host, without introducing other undesired genes. The availability of efficient gene transfer procedures in grapevine genotypes provide valid tools that support the application of new breeding techniques (NBTs). The expertise built up over the years has allowed the optimization of these techniques to overexpress genes that directly or indirectly limit fungal and oomycetes pathogens growth or silence plant susceptibility genes. Furthermore, the downregulation of pathogen genes which act as virulence effectors by exploiting the RNA interference mechanism, represents another biotechnological tool that increases plant defense. In this review, we summarize the most recent biotechnological strategies optimized and applied on Vitis species, aimed at reducing their susceptibility to the most harmful fungal and oomycetes diseases. The best strategy for combating pathogenic organisms is to exploit a holistic approach that fully integrates all these available tools.

Chen, H., et al. (2018). "Aptazyme-mediated direct modulation of post-transcriptional sgRNA level for conditional genome editing and gene expression." J Biotechnol **288**: 23-29.

RNA-guided endonuclease Cas9 derived from microbial CRISPR-Cas adaptive immune systems is a powerful tool for genome editing, which has been widely used in eukaryotic systems, prokaryotic systems, and plants. However, the off-target effects caused by Cas9/sgRNA remain a major concern. Currently, the efforts to reduce the off-target effects mainly focus on improving the targeting specificity of sgRNA/Cas9, regulating the activity of the Cas9 protein or the sgRNA, and controlling the time window of their expression. In this study, a novel system was established to regulate the post-transcriptional sgRNA level by small molecule-controlled aptazyme. This system was shown to reduce the off-target effects caused by Cas9/sgRNA, while enabling precise temporal control over gene editing and regulatory activity. This new system could provide a potentially safer and more powerful tool for genome editing and therapeutic application.

Choi, W., et al. (2015). "Efficient PRNP deletion in bovine genome using gene-editing technologies in bovine cells." Prion **9**(4): 278-291.

Even though prion (encoded by the PRNP gene) diseases like bovine spongiform encephalopathy

(BSE) are fatal neurodegenerative diseases in cattle, their study via gene deletion has been limited due to the absence of cell lines or mutant models. In this study, we aim to develop an immortalized fibroblast cell line in which genome-engineering technology can be readily applied to create gene-modified clones for studies. To this end, this study is designed to 1) investigate the induction of primary fibroblasts to immortalization by introducing Bmi-1 and hTert genes; 2) investigate the disruption of the PRNP in those cells; and 3) evaluate the gene expression and embryonic development using knockout (KO) cell lines. Primary cells from a male neonate were immortalized with Bmi-1and hTert. Immortalized cells were cultured for more than 180 days without any changes in their doubling time and morphology. Furthermore, to knockout the PRNP gene, plasmids that encode transcription activator-like effector nuclease (TALEN) pairs were transfected into the cells, and transfected single cells were propagated. Mutated clonal cell lines were confirmed by T7 endonuclease I assay and sequencing. Four knockout cell lines were used for somatic cell nuclear transfer (SCNT), and the resulting embryos were developed to the blastocyst stage. The genes (CSNK2A1, FAM64A, MPG and PRND) were affected after PRNP disruption in immortalized cells. In conclusion, we established immortalized cattle fibroblasts using Bmi-1 and hTert genes, and used TALENs to knockout the PRNP gene in these immortalized cells. The efficient PRNP KO is expected to be a useful technology to develop our understanding of in vitro prion protein functions in cattle.

Conway, A., et al. (2019). "Non-viral Delivery of Zinc Finger Nuclease mRNA Enables Highly Efficient In Vivo Genome Editing of Multiple Therapeutic Gene Targets." Mol Ther **27**(4): 866-877.

It has previously been shown that engineered zinc finger nucleases (ZFNs) can be packaged into adeno-associated viruses (AAVs) and delivered intravenously into mice, non-human primates, and most recently, humans to induce highly efficient therapeutic genome editing in the liver. Lipid nanoparticles (LNPs) are synthetic delivery vehicles that enable repeat administration and are not limited by the presence of preexisting neutralizing antibodies in patients. Here, we show that mRNA encoding ZFNs formulated into LNP can enable >90% knockout of gene expression in mice by targeting the TTR or PCSK9 gene, at mRNA doses 10-fold lower than has ever been reported. Additionally, co-delivering mRNA-LNP containing ZFNs targeted to intron 1 of the ALB locus with AAV packaged with a promoterless human IDS or FIX therapeutic transgene can result in high levels of targeted integration and subsequent therapeutically relevant levels of protein expression in mice. Finally, we show repeat administration of ZFN mRNA-LNP after a single AAV donor dose results in significantly increased levels of genome editing and transgene expression compared to a single dose. These results demonstrate LNP-mediated ZFN mRNA delivery can drive highly efficient levels of in vivo genome editing and can potentially offer a new treatment modality for a variety of diseases.

Cui, Z., et al. (2017). "Genome editing reveals dmrt1 as an essential male sex-determining gene in Chinese tongue sole (Cynoglossus semilaevis)." Sci Rep **7**: 42213.

Chinese tongue sole is a marine fish with ZW sex determination. Genome sequencing suggested that the Z-linked dmrt1 is a putative male determination gene, but direct genetic evidence is still lacking. Here we show that TALEN of dmrt1 efficiently induced mutations of this gene. The ZZ dmrt1 mutant fish developed ovary-like testis, and the spermatogenesis was disrupted. The female-related genes foxl2 and cyp19a1a were significantly increased in the gonad of the ZZ dmrt1 mutant. Conversely, the male-related genes Sox9a and Amh were significantly decreased. The dmrt1 deficient ZZ fish grew much faster than ZZ male control. Notably, we obtained an intersex ZW fish with a testis on one side and an ovary on the other side. This fish was chimeric for a dmrt1 mutation in the ovary, and wild-type dmrt1 in the testis. Our data provide the first functional evidence that dmrt1 is a male determining gene in tongue sole.

Czerwinska, P., et al. (2019). "Gene delivery methods and genome editing of human pluripotent stem cells." Rep Pract Oncol Radiother **24**(2): 180-187.

Induced pluripotent stem cells derived from normal somatic cells could be utilized to study tumorigenesis through overexpression of specific oncogenes, downregulation of tumor suppressors and dysregulation of other factors thought to promote tumorigenesis. Therefore, effective approaches that provide direct modifications of induced pluripotent stem cell genome are extremely needed. Emerging strategies are expected to provide the ability to more effectively introduce diverse genetic alterations, from as small as single-nucleotide modifications to whole gene amplification or deletion, all with a high degree of target specificity. To date, several techniques have been applied in stem cell studies to directly edit cell genome (ZFNs, TALENs or CRISPR/Cas9). In this review, we summarize specific gene delivery strategies that were applied to stem cell studies together with genome editing techniques, which enable a direct modification of endogenous DNA sequences in the context of cancer studies.

Duan, J. (2015). "Path from schizophrenia genomics to biology: gene regulation and perturbation in neurons derived from induced pluripotent stem cells and genome editing." Neurosci Bull **31**(1): 113-127.

Schizophrenia (SZ) is a devastating mental disorder afflicting 1% of the population. Recent genome-wide association studies (GWASs) of SZ have identified >100 risk loci. However, the causal variants/genes and the causal mechanisms remain largely unknown, which hinders the translation of GWAS findings into disease biology and drug targets. Most risk variants are noncoding, thus likely regulate gene expression. A major mechanism of transcriptional regulation is chromatin remodeling, and open chromatin is a versatile predictor of regulatory sequences. MicroRNA-mediated post-transcriptional regulation plays an important role in SZ pathogenesis. Neurons differentiated from patient-specific induced pluripotent stem cells (iPSCs) provide an experimental model to characterize the genetic perturbation of regulatory variants that are often specific to cell type and/or developmental stage. The emerging genome-editing technology enables the creation of isogenic iPSCs and neurons to efficiently characterize the effects of SZ-associated regulatory variants on SZ-relevant molecular and cellular phenotypes involving dopaminergic, glutamatergic, and GABAergic neurotransmissions. SZ GWAS findings equipped with the emerging functional genomics approaches provide an unprecedented opportunity for understanding new disease biology and identifying novel drug targets.

Elliott, E. K., et al. (2021). "Mini review: genome and transcriptome editing using CRISPR-cas systems for haematological malignancy gene therapy." Transgenic Res **30**(2): 129-141.

The recent introduction of clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR associated protein (Cas) systems, offer an array of genome and transcriptome editing tools for clinical repair strategies. These include Cas9, Cas12a, dCas9 and more recently Cas13 effectors. RNA targeting CRISPR-Cas13 complexes show unique characteristics with the capability to engineer transcriptomes and modify gene expression, providing a potential clinical cancer therapy tool across various tissue types. Cas13 effectors such as RNA base editing for A to I replacement allows for precise transcript modification. Further applications of Cas13a highlights its capability of producing rapid diagnostic results in a mobile platform. This review will focus on the adaptions of existing CRISPR-Cas systems, along with new Cas effectors for transcriptome or RNA modifications used in disease modelling and gene therapy for haematological malignancy. We also address the current diagnostic and therapeutic potential of CRISPR-Cas systems for personalised haematological malignancy.

Endo, M., et al. (2021). "Precision genome editing in plants via gene targeting and subsequent break-induced single-strand annealing." Plant Biotechnol J **19**(3): 563-574.

Genome editing via artificial nucleases such as CRISPR/Cas9 has become popular in plants now. However, small insertions or deletions are major mutations and nucleotide substitutions rarely occur when DNA cleavage is induced. To induce nucleotide substitutions, a base editor utilizing dead or nickase-type Cas9 fused with deaminase have been developed. However, the direction and position of practical substitution are still limited. In this context, homologous recombination (HR)-mediated gene targeting (GT) has advantages because any mutations existing on the donor DNA are copied and passed onto the endogenous DNA. As HR-mediated GT is extremely rare in higher plants, positive-negative selection has been used to isolate cells in which GT has occurred. After successful selection, positive selection marker is no longer needed and should ideally be eliminated. In a previous study, we reported a seamless piggyBac-transposon-mediated marker elimination system. Precision marker elimination efficiency in this system is very high. The piggyBac transposon integrates into the host genome at TTAA elements and excises without leaving a footprint at the excised site, so a TTAA sequence is necessary at the location of a positive selection marker. To compensate for this limitation, we have developed a novel marker elimination system using an I-SceI break and subsequent single-strand annealing (SSA)-mediated DNA repair system.

Farasat, I. and H. M. Salis (2016). "A Biophysical Model of CRISPR/Cas9 Activity for Rational Design of Genome Editing and Gene Regulation." PLoS Comput Biol **12**(1): e1004724.

The ability to precisely modify genomes and regulate specific genes will greatly accelerate several medical and engineering applications. The CRISPR/Cas9 (Type II) system binds and cuts DNA using guide RNAs, though the variables that control its on-target and off-target activity remain poorly characterized. Here, we develop and parameterize a system-wide biophysical model of Cas9-based genome editing and gene regulation to predict how changing guide RNA sequences, DNA superhelical densities, Cas9 and crRNA expression levels, organisms and growth conditions, and experimental conditions collectively control the dynamics of dCas9-based binding and Cas9-based cleavage at all DNA sites with both canonical and non-canonical PAMs. We combine

statistical thermodynamics and kinetics to model Cas9:crRNA complex formation, diffusion, site selection, reversible R-loop formation, and cleavage, using large amounts of structural, biochemical, expression, and next-generation sequencing data to determine kinetic parameters and develop free energy models. Our results identify DNA supercoiling as a novel mechanism controlling Cas9 binding. Using the model, we predict Cas9 off-target binding frequencies across the lambdaphage and human genomes, and explain why Cas9's off-target activity can be so high. With this improved understanding, we propose several rules for designing experiments for minimizing off-target activity. We also discuss the implications for engineering dCas9-based genetic circuits.

Ferreira, R., et al. (2018). "Multiplexed CRISPR/Cas9 Genome Editing and Gene Regulation Using Csy4 in Saccharomyces cerevisiae." ACS Synth Biol **7**(1): 10-15.

Clustered regularly interspaced short palindromic repeats (CRISPR) technology has greatly accelerated the field of strain engineering. However, insufficient efforts have been made toward developing robust multiplexing tools in Saccharomyces cerevisiae. Here, we exploit the RNA processing capacity of the bacterial endoribonuclease Csy4 from Pseudomonas aeruginosa, to generate multiple gRNAs from a single transcript for genome editing and gene interference applications in S. cerevisiae. In regards to genome editing, we performed a quadruple deletion of FAA1, FAA4, POX1 and TES1 reaching 96% efficiency out of 24 colonies tested. Then, we used this system to efficiently transcriptionally regulate the three genes, OLE1, HMG1 and ACS1. Thus, we demonstrate that multiplexed genome editing and gene regulation can be performed in a fast and effective manner using Csy4.

Fujii, T., et al. (2018). "Translucent larval integument and flaccid paralysis caused by genome editing in a gene governing molybdenum cofactor biosynthesis in Bombyx mori." Insect Biochem Mol Biol **99**: 11-16.

Translucency of the larval integument in Bombyx mori is caused by a lack of uric acid in the epidermis. Hime'nichi translucent (ohi) is a unique mutation causing intermediate translucency of the larval integument and male-specific flaccid paralysis. To determine the gene associated with the ohi mutation, the ohi locus was mapped to a 400-kb region containing 29 predicted genes. Among the genes in this region, we focused on Bombyx homolog of mammalian Gephyrin (BmGphn), which regulates molybdenum cofactor (MoCo) biosynthesis, because MoCo is indispensable for the activity of xanthine dehydrogenase (XDH), a key enzyme in uric acid biosynthesis. The translucent integument of ohi larvae turned opaque after injection of bovine xanthine oxidase, which is a mammalian equivalent to XDH, indicating that XDH activity is defective in ohi larvae. RT-PCR and sequencing analysis showed that (i) in ohi larvae, expression of the BmGphn gene was repressed in the fat body where uric acid is synthesized, and (ii) there was no amino acid substitution in the ohi mutant allele. Finally, we obtained BmGphn knockout alleles (hereafter denoted as BmGphn(Delta)) by using CRISPR/Cas9. The resulting ohi/BmGphn(Delta) larvae had translucent integuments, demonstrating that BmGphn is the gene responsible for the ohi phenotype. Our results show that repressed expression of BmGphn is a causative factor for the defective MoCo biosynthesis and XDH activity observed in ohi larvae. Interestingly, all male BmGphn(Delta) homozygotes died before pupation and showed a flaccid paralysis phenotype. The genetic and physiological mechanisms underlying this flaccid paralysis phenotype are also discussed.

Gaj, T., et al. (2017). "Targeted gene knock-in by homology-directed genome editing using Cas9 ribonucleoprotein and AAV donor delivery." Nucleic Acids Res **45**(11): e98.

Realizing the full potential of genome editing requires the development of efficient and broadly applicable methods for delivering programmable nucleases and donor templates for homology-directed repair (HDR). The RNA-guided Cas9 endonuclease can be introduced into cells as a purified protein in complex with a single guide RNA (sgRNA). Such ribonucleoproteins (RNPs) can facilitate the high-fidelity introduction of single-base substitutions via HDR following co-delivery with a single-stranded DNA oligonucleotide. However, combining RNPs with transgene-containing donor templates for targeted gene addition has proven challenging, which in turn has limited the capabilities of the RNP-mediated genome editing toolbox. Here, we demonstrate that combining RNP delivery with naturally recombinogenic adeno-associated virus (AAV) donor vectors enables site-specific gene insertion by homology-directed genome editing. Compared to conventional plasmid-based expression vectors and donor templates, we show that combining RNP and AAV donor delivery increases the efficiency of gene addition by up to 12-fold, enabling the creation of lineage reporters that can be used to track the conversion of striatal neurons from human fibroblasts in real time. These results thus illustrate the potential for unifying nuclease protein delivery with AAV donor vectors for homology-directed genome editing.

Gao, S., et al. (2016). "Multiplex gene editing of the Yarrowia lipolytica genome using the CRISPR-Cas9 system." J Ind Microbiol Biotechnol **43**(8): 1085-1093.

Yarrowia lipolytica is categorized as a generally recognized as safe (GRAS) organism and is a heavily documented, unconventional yeast that has been widely incorporated into multiple industrial fields to produce valuable biochemicals. This study describes the construction of a CRISPR-Cas9 system for genome editing in Y. lipolytica using a single plasmid (pCAS1yl or pCAS2yl) to transport Cas9 and relevant guide RNA expression cassettes, with or without donor DNA, to target genes. Two Cas9 target genes, TRP1 and PEX10, were repaired by non-homologous end-joining (NHEJ) or homologous recombination, with maximal efficiencies in Y. lipolytica of 85.6 % for the wild-type strain and 94.1 % for the ku70/ku80 double-deficient strain, within 4 days. Simultaneous double and triple multigene editing was achieved with pCAS1yl by NHEJ, with efficiencies of 36.7 or 19.3 %, respectively, and the pCASyl system was successfully expanded to different Y. lipolytica breeding strains. This timesaving method will enable and improve synthetic biology, metabolic engineering and functional genomic studies of Y. lipolytica.

Gee, P., et al. (2017). "Cellular Reprogramming, Genome Editing, and Alternative CRISPR Cas9 Technologies for Precise Gene Therapy of Duchenne Muscular Dystrophy." Stem Cells Int **2017**: 8765154.

In the past decade, the development of two innovative technologies, namely, induced pluripotent stem cells (iPSCs) and the CRISPR Cas9 system, has enabled researchers to model diseases derived from patient cells and precisely edit DNA sequences of interest, respectively. In particular, Duchenne muscular dystrophy (DMD) has been an exemplary monogenic disease model for combining these technologies to demonstrate that genome editing can correct genetic mutations in DMD patient-derived iPSCs. DMD is an X-linked genetic disorder caused by mutations that disrupt the open reading frame of the dystrophin gene, which plays a critical role in stabilizing muscle cells during contraction and relaxation. The CRISPR Cas9 system has been shown to be capable of targeting the dystrophin gene and rescuing its expression in in vitro patient-derived iPSCs and in vivo DMD mouse models. In this review, we highlight recent advances made using the CRISPR Cas9 system to correct genetic mutations and discuss how emerging CRISPR technologies and iPSCs in a combined platform can play a role in bringing a therapy for DMD closer to the clinic.

Gonen, S., et al. (2017). "Potential of gene drives with genome editing to increase genetic gain in livestock breeding programs." Genet Sel Evol **49**(1): 3.

BACKGROUND: This paper uses simulation to explore how gene drives can increase genetic gain in livestock breeding programs. Gene drives are naturally occurring phenomena that cause a mutation on one chromosome to copy itself onto its homologous chromosome. METHODS: We simulated nine different breeding and editing scenarios with a common overall structure. Each scenario began with 21 generations of selection, followed by 20 generations of selection based on true breeding values where the breeder used selection alone, selection in combination with genome editing, or selection with genome editing and gene drives. In the scenarios that used gene drives, we varied the probability of successfully incorporating the gene drive. For each scenario, we evaluated genetic gain, genetic variance [Formula: see text], rate of change in inbreeding ([Formula: see text]), number of distinct quantitative trait nucleotides (QTN) edited, rate of increase in favourable allele frequencies of edited QTN and the time to fix favourable alleles. RESULTS: Gene drives enhanced the benefits of genome editing in seven ways: (1) they amplified the increase in genetic gain brought about by genome editing; (2) they amplified the rate of increase in the frequency of favourable alleles and reduced the time it took to fix them; (3) they enabled more rapid targeting of QTN with lesser effect for genome editing; (4) they distributed fixed editing resources across a larger number of distinct QTN across generations; (5) they focussed editing on a smaller number of QTN within a given generation; (6) they reduced the level of inbreeding when editing a subset of the sires; and (7) they increased the efficiency of converting genetic variation into genetic gain. CONCLUSIONS: Genome editing in livestock breeding results in short-, medium- and long-term increases in genetic gain. The increase in genetic gain occurs because editing increases the frequency of favourable alleles in the population. Gene drives accelerate the increase in allele frequency caused by editing, which results in even higher genetic gain over a shorter period of time with no impact on inbreeding.

Gori, J. L., et al. (2015). "Delivery and Specificity of CRISPR-Cas9 Genome Editing Technologies for Human Gene Therapy." Hum Gene Ther **26**(7): 443-451.

Genome editing using the clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR associated 9 (Cas9) technology is revolutionizing the study of gene function and likely will give rise to an entire new class of therapeutics for a wide range of diseases. Achieving this goal requires

not only characterization of the technology for efficacy and specificity but also optimization of its delivery to the target cells for each disease indication. In this review we survey the various methods by which the CRISPR-Cas9 components have been delivered to cells and highlight some of the more clinically relevant approaches. Additionally, we discuss the methods available for assessing the specificity of Cas9 editing; an important safety consideration for development of the technology.

Grosche, C., et al. (2012). "The chloroplast genome of Pellia endiviifolia: gene content, RNA-editing pattern, and the origin of chloroplast editing." Genome Biol Evol **4**(12): 1349-1357.

RNA editing is a post-transcriptional process that can act upon transcripts from mitochondrial, nuclear, and chloroplast genomes. In chloroplasts, single-nucleotide conversions in mRNAs via RNA editing occur at different frequencies across the plant kingdom. These range from several hundred edited sites in some mosses and ferns to lower frequencies in seed plants and the complete lack of RNA editing in the liverwort Marchantia polymorpha. Here, we report the sequence and edited sites of the chloroplast genome from the liverwort Pellia endiviifolia. The type and frequency of chloroplast RNA editing display a pattern highly similar to that in seed plants. Analyses of the C to U conversions and the genomic context in which the editing sites are embedded provide evidence in favor of the hypothesis that chloroplast RNA editing evolved to compensate mutations in the first land plants.

Han, Y., et al. (2015). "CRISPR-Cas9 genome editing of a single regulatory element nearly abolishes target gene expression in mice--brief report." Arterioscler Thromb Vasc Biol **35**(2): 312-315.

OBJECTIVE: To ascertain the importance of a single regulatory element in the control of Cnn1 expression using CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9) genome editing. APPROACH AND RESULTS: The CRISPR/Cas9 system was used to produce 3 of 18 founder mice carrying point mutations in an intronic CArG box of the smooth muscle cell-restricted Cnn1 gene. Each founder was bred for germline transmission of the mutant CArG box and littermate interbreeding to generate homozygous mutant (Cnn1(DeltaCArG/DeltaCArG)) mice. Quantitative reverse transcription polymerase chain reaction, Western blotting, and confocal immunofluorescence microscopy showed dramatic reductions in Cnn1 mRNA and CNN1 protein expression in Cnn1(DeltaCArG/DeltaCArG) mice with no change in other smooth muscle cell-restricted genes and little evidence of off-target edits elsewhere in the genome. In vivo chromatin immunoprecipitation assay revealed a sharp decrease in binding of serum response factor to the mutant CArG box. Loss of CNN1 expression was coincident with an increase in Ki-67 positive cells in the normal vessel wall. CONCLUSIONS: CRISPR/Cas9 genome editing of a single CArG box nearly abolishes Cnn1 expression in vivo and evokes increases in smooth muscle cell DNA synthesis. This facile genome editing system paves the way for a new generation of studies designed to test the importance of individual regulatory elements in living animals, including regulatory variants in conserved sequence blocks linked to human disease.

Hao, F., et al. (2018). "Generation of Cashmere Goats Carrying an EDAR Gene Mutant Using CRISPR-Cas9-Mediated Genome Editing." Int J Biol Sci **14**(4): 427-436.

In recent years, while the use of the clustered regularly interspaced short palindromic repeat (CRISPR)-CRISPR-associated protein 9 (Cas9) (CRISPR-Cas9) system for targeted genome editing has become a research hotspot, it has, to date, not proved adequate for genome editing in large mammals, such as goats. In this study, two opposite single-guide RNAs (sgRNAs) were designed for complete EDAR gene targeting in Cashmere goats, and co-transfected with a plasmid encoding Cas9 into goat fibroblasts. Among the 89 cell lines obtained through the cultivation of clonal cell lines, 62 were positive for EDAR gene targeting. Nine types of mutations were identified by sequencing analysis, and the mutation efficiency was 69.7%. Using one of these cell lines, EDAR gene-targeted Cashmere goat embryos were prepared by somatic cell cloning. Developed embryos were transferred to 79 Cashmere goat recipients, and, after a gestation period of five months six male EDAR gene-targeted Cashmere goats were born. Although only two of these goats survived, they had abnormal primary hair follicles and no hair on the top of their heads, which are the distinctive features of the EDAR gene-targeted Cashmere goats. Thus, this study provides a valuable animal model for future studies on EDAR gene-related phenotypes and hair follicle growth and development and shows that the CRISPR-Cas9 system can be used to edit genes in large mammals.

He, Z., et al. (2016). "Comparison of CRISPR/Cas9 and TALENs on editing an integrated EGFP gene in the genome of HEK293FT cells." Springerplus **5**(1): 814.

BACKGROUND: Genome editors such as CRISPR/Cas9 and TALENs are at the forefront of research into methodologies for targeted modification of the mammalian genome. To date few comparative

studies have been carried out to investigate the difference of genome editing characteristics between CRISPR/Cas9 and TALENs. While the CRISPR/Cas9 system has overtaken TALENs as the tool of choice for most research groups working in this field, we hypothesized that there could be certain applications whereby the application of TALENs would have specific benefits. Here we compare CRISPR/Cas9 and TALEN as tools for introducing site-specific editing events at an integrated EGFP gene in the genome of HEK293FT cells. RESULTS: Guide RNAs and TALEN pairs were designed to target two loci within the EGFP gene. We found that paired Cas9 nucleases induced targeted genomic deletion more efficiently and precisely than two TALEN pairs. However, when concurrently supplied with a plasmid template spanning the two DNA double-strand breaks (DSBs) within EGFP, TALENs stimulated homology directed repair (HDR) more efficiently than CRISPR/Cas9 and caused fewer targeted genomic deletions. CONCLUSIONS: Our data suggest that the choice of genome editing tool should be determined by the desired genome editing outcome. Such a rational approach is likely to benefit research outputs for groups working in fields as diverse as modification of cell lines, to animal models for disease studies, or gene therapy strategies.

Hirata, M., et al. (2019). "Genome mutation after introduction of the gene editing by electroporation of Cas9 protein (GEEP) system in matured oocytes and putative zygotes." In Vitro Cell Dev Biol Anim **55**(4): 237-242.

The application of CRISPR/Cas9 strategy promises to rapidly increase the production of genetically engineered animals since it yields stably integrated transgenes. In the present study, we investigated the efficiency of target mutations after electroporation with the CRISPR/Cas9 system using sgRNAs to target the MSTN or FGF10 genes in porcine-matured oocytes and putative zygotes. Effects of pulse number (3-7 pulse repetitions) during electroporation on the embryonic development and mutation efficiency were also investigated. Our results showed that the cleavage rate of matured oocytes with electroporation treatment significantly decreased as compared with electroporated putative zygotes ($p < 0.05$). Moreover, the rates of blastocyst formation from oocytes/zygotes electroporated with more than 5 pulses decreased. Mutation efficiency was then assessed after sequencing the target sites in individual blastocysts derived from oocytes/zygotes electroporated by 3 and 5 pulses. No bi-allelic mutations in all examined blastocysts were observed in this study. There were no differences in the mutation rates (50-60%) between blastocysts derived from matured oocytes electroporated by 3 and 5 pulses, irrespective of targeting gene. In the targeting MSTN gene, however, the mutation rate (12.5%) of blastocysts derived from putative zygotes electroporated by 3 pulses tended to be lower than that (60%) from 5-pulsed electroporated putative zygotes. These data indicate that the type of eggs may influence not only their development after electroporation treatment but also the mutation rate in the resulting blastocysts.

Hirose, M., et al. (2017). "CRISPR/Cas9-mediated genome editing in wild-derived mice: generation of tamed wild-derived strains by mutation of the a (nonagouti) gene." Sci Rep **7**: 42476.

Wild-derived mice have contributed to experimental mouse genetics by virtue of their genetic diversity, which may help increase the chance of identifying novel modifier genes responsible for specific phenotypes and diseases. However, gene targeting using wild-derived mice has been unsuccessful because of the unavailability of stable embryonic stem cells. Here, we report that CRISPR/Cas9-mediated gene targeting can be applied to the Japanese wild-derived MSM/Ms strain (Mus musculus molossinus). We targeted the nonagouti (a) gene encoding the agouti protein that is localized in hair and the brain. We obtained three homozygous knockout mice as founders, all showing black coat colour. While homozygous knockout offspring were physiologically indistinguishable from wild-type litter-mates, they showed specific domesticated behaviours: hypoactivity in the dark phase and a decline in the avoidance of a human hand. These phenotypes were consistent over subsequent generations. Our findings support the empirical hypothesis that nonagouti is a domestication-linked gene, the loss of which might repress aggressive behaviour.

Hotta, A. (2015). "Genome Editing Gene Therapy for Duchenne Muscular Dystrophy." J Neuromuscul Dis **2**(4): 343-355.

Duchenne muscular dystrophy (DMD) is a severe genetic disorder caused by loss of function of the dystrophin gene on the X chromosome. Gene augmentation of dystrophin is challenging due to the large size of the dystrophin cDNA. Emerging genome editing technologies, such as TALEN and CRISPR-Cas9 systems, open a new erain the restoration of functional dystrophin and are a hallmark of bona fide gene therapy. In this review, we summarize current genome editing approaches, properties of target cell types for ex vivo gene therapy, and perspectives of in vivo gene therapy including genome editing in human zygotes. Although technical challenges, such as efficacy, accuracy, and delivery of the genome editing components, remain to be further improved, yet

genome editing technologies offer a new avenue for the gene therapy of DMD.

Hotta, A. and S. Yamanaka (2015). "From Genomics to Gene Therapy: Induced Pluripotent Stem Cells Meet Genome Editing." Annu Rev Genet **49**: 47-70.

The advent of induced pluripotent stem (iPS) cells has opened up numerous avenues of opportunity for cell therapy, including the initiation in September 2014 of the first human clinical trial to treat dry age-related macular degeneration. In parallel, advances in genome-editing technologies by site-specific nucleases have dramatically improved our ability to edit endogenous genomic sequences at targeted sites of interest. In fact, clinical trials have already begun to implement this technology to control HIV infection. Genome editing in iPS cells is a powerful tool and enables researchers to investigate the intricacies of the human genome in a dish. In the near future, the groundwork laid by such an approach may expand the possibilities of gene therapy for treating congenital disorders. In this review, we summarize the exciting progress being made in the utilization of genomic editing technologies in pluripotent stem cells and discuss remaining challenges toward gene therapy applications.

Hu, M., et al. (2020). "Functional Genomics in Pancreatic beta Cells: Recent Advances in Gene Deletion and Genome Editing Technologies for Diabetes Research." Front Endocrinol (Lausanne) **11**: 576632.

The inheritance of variants that lead to coding changes in, or the mis-expression of, genes critical to pancreatic beta cell function can lead to alterations in insulin secretion and increase the risk of both type 1 and type 2 diabetes. Recently developed clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) gene editing tools provide a powerful means of understanding the impact of identified variants on cell function, growth, and survival and might ultimately provide a means, most likely after the transplantation of genetically "corrected" cells, of treating the disease. Here, we review some of the disease-associated genes and variants whose roles have been probed up to now. Next, we survey recent exciting developments in CRISPR/Cas9 technology and their possible exploitation for beta cell functional genomics. Finally, we will provide a perspective as to how CRISPR/Cas9 technology may find clinical application in patients with diabetes.

Huang, X., et al. (2015). "Production of Gene-Corrected Adult Beta Globin Protein in Human Erythrocytes Differentiated from Patient iPSCs After Genome Editing of the Sickle Point Mutation." Stem Cells **33**(5): 1470-1479.

Human induced pluripotent stem cells (iPSCs) and genome editing provide a precise way to generate gene-corrected cells for disease modeling and cell therapies. Human iPSCs generated from sickle cell disease (SCD) patients have a homozygous missense point mutation in the HBB gene encoding adult beta-globin proteins, and are used as a model system to improve strategies of human gene therapy. We demonstrate that the CRISPR/Cas9 system designer nuclease is much more efficient in stimulating gene targeting of the endogenous HBB locus near the SCD point mutation in human iPSCs than zinc finger nucleases and TALENs. Using a specific guide RNA and Cas9, we readily corrected one allele of the SCD HBB gene in human iPSCs by homologous recombination with a donor DNA template containing the wild-type HBB DNA and a selection cassette that was subsequently removed to avoid possible interference of HBB transcription and translation. We chose targeted iPSC clones that have one corrected and one disrupted SCD allele for erythroid differentiation assays, using an improved xeno-free and feeder-free culture condition we recently established. Erythrocytes from either the corrected or its parental (uncorrected) iPSC line were generated with similar efficiencies. Currently approximately 6%-10% of these differentiated erythrocytes indeed lacked nuclei, characteristic of further matured erythrocytes called reticulocytes. We also detected the 16-kDa beta-globin protein expressed from the corrected HBB allele in the erythrocytes differentiated from genome-edited iPSCs. Our results represent a significant step toward the clinical applications of genome editing using patient-derived iPSCs to generate disease-free cells for cell and gene therapies. Stem Cells 2015;33:1470-1479.

Huhn, S. C., et al. (2019). "High throughput, efficacious gene editing & genome surveillance in Chinese hamster ovary cells." PLoS One **14**(12): e0218653.

Chinese hamster ovary (CHO) cells are a common tool utilized in bioproduction and directed genome engineering of CHO cells is of great interest to enhance recombinant cell lines. Until recently, this focus has been challenged by a lack of efficacious, high throughput, and low-cost gene editing modalities and screening methods. In this work, we demonstrate an improved method for gene editing in CHO cells using CRISPR RNPs and characterize the endpoints of Cas9 and ZFN mediated genetic engineering. Furthermore, we validate sequence decomposition as a cost effective, rapid, and accurate method for assessing mutants and eliminating non-clonal CHO populations using only capillary sequencing.

Jervis, A. J., et al. (2021). "A plasmid toolset for CRISPR-mediated genome editing and CRISPRi gene regulation in Escherichia coli." Microb Biotechnol **14**(3): 1120-1129.

CRISPR technologies have become standard laboratory tools for genetic manipulations across all kingdoms of life. Despite their origins in bacteria, the development of CRISPR tools for engineering bacteria has been slower than for eukaryotes; nevertheless, their function and application for genome engineering and gene regulation via CRISPR interference (CRISPRi) has been demonstrated in various bacteria, and adoption has become more widespread. Here, we provide simple plasmid-based systems for genome editing (gene knockouts/knock-ins, and genome integration of large DNA fragments) and CRISPRi in E. coli using a CRISPR-Cas12a system. The described genome engineering protocols allow markerless deletion or genome integration in just seven working days with high efficiency (> 80% and 50%, respectively), and the CRISPRi protocols allow robust transcriptional repression of target genes (> 90%) with a single cloning step. The presented minimized plasmids and their associated design and experimental protocols provide efficient and effective CRISPR-Cas12 genome editing, genome integration and CRISPRi implementation. These simple-to-use systems and protocols will allow the easy adoption of CRISPR technology by any laboratory.

Jia, H., et al. (2017). "Genome editing of the disease susceptibility gene CsLOB1 in citrus confers resistance to citrus canker." Plant Biotechnol J **15**(7): 817-823.

Citrus is a highly valued tree crop worldwide, while, at the same time, citrus production faces many biotic challenges, including bacterial canker and Huanglongbing (HLB). Breeding for disease-resistant varieties is the most efficient and sustainable approach to control plant diseases. Traditional breeding of citrus varieties is challenging due to multiple limitations, including polyploidy, polyembryony, extended juvenility and long crossing cycles. Targeted genome editing technology has the potential to shorten varietal development for some traits, including disease resistance. Here, we used CRISPR/Cas9/sgRNA technology to modify the canker susceptibility gene CsLOB1 in Duncan grapefruit. Six independent lines, DLOB 2, DLOB 3, DLOB 9, DLOB 10, DLOB 11 and DLOB 12, were generated. Targeted next-generation sequencing of the six lines showed the mutation rate was 31.58%, 23.80%, 89.36%, 88.79%, 46.91% and 51.12% for DLOB 2, DLOB 3, DLOB 9, DLOB 10, DLOB 11 and DLOB 12, respectively, of the cells in each line. DLOB 2 and DLOB 3 showed canker symptoms similar to wild-type grapefruit, when inoculated with the pathogen Xanthomonas citri subsp. citri (Xcc). No canker symptoms were observed on DLOB 9, DLOB 10, DLOB 11 and DLOB 12 at 4 days postinoculation (DPI) with Xcc. Pustules caused by Xcc were observed on DLOB 9, DLOB 10, DLOB 11 and DLOB 12 in later stages, which were much reduced compared to that on wild-type grapefruit. The pustules on DLOB 9 and DLOB 10 did not develop into typical canker symptoms. No side effects and off-target mutations were detected in the mutated plants. This study indicates that genome editing using CRISPR technology will provide a promising pathway to generate disease-resistant citrus varieties.

Jiang, N., et al. (2019). "Development of Beet necrotic yellow vein virus-based vectors for multiple-gene expression and guide RNA delivery in plant genome editing." Plant Biotechnol J **17**(7): 1302-1315.

Many plant viruses with monopartite or bipartite genomes have been developed as efficient expression vectors of foreign recombinant proteins. Nonetheless, due to lack of multiple insertion sites in these plant viruses, it is still a big challenge to simultaneously express multiple foreign proteins in single cells. The genome of Beet necrotic yellow vein virus (BNYVV) offers an attractive system for expression of multiple foreign proteins owning to a multipartite genome composed of five positive-stranded RNAs. Here, we have established a BNYVV full-length infectious cDNA clone under the control of the Cauliflower mosaic virus 35S promoter. We further developed a set of BNYVV-based vectors that permit efficient expression of four recombinant proteins, including some large proteins with lengths up to 880 amino acids in the model plant Nicotiana benthamiana and native host sugar beet plants. These vectors can be used to investigate the subcellular co-localization of multiple proteins in leaf, root and stem tissues of systemically infected plants. Moreover, the BNYVV-based vectors were used to deliver NbPDS guide RNAs for genome editing in transgenic plants expressing Cas9, which induced a photobleached phenotype in systemically infected leaves. Collectively, the BNYVV-based vectors will facilitate genomic research and expression of multiple proteins, in sugar beet and related crop plants.

Katayama, T., et al. (2019). "Forced Recycling of an AMA1-Based Genome-Editing Plasmid Allows for Efficient Multiple Gene Deletion/Integration in the Industrial Filamentous Fungus Aspergillus oryzae." Appl Environ Microbiol **85**(3).

Filamentous fungi are used for food fermentation and industrial production of recombinant proteins. They also serve as a source of secondary metabolites and are recently expected as hosts for

heterologous production of useful secondary metabolites. Multiple-step genetic engineering is required to enhance industrial production involving these fungi, but traditional sequential modification of multiple genes using a limited number of selection markers is laborious. Moreover, efficient genetic engineering techniques for industrial strains have not yet been established. We have previously developed a clustered regulatory interspaced short palindromic repeats (CRISPR)/Cas9-based mutagenesis technique for the industrial filamentous fungus Aspergillus oryzae, enabling mutation efficiency of 10 to 20%. Here, we improved the CRISPR/Cas9 approach by including an AMA1-based autonomously replicating plasmid harboring the drug resistance marker ptrA By using the improved mutagenesis technique, we successfully modified A. oryzae wild and industrial strains, with a mutation efficiency of 50 to 100%. Conditional expression of the Aoace2 gene from the AMA1-based plasmid severely inhibited fungal growth. This enabled forced recycling of the plasmid, allowing repeated genome editing. Further, double mutant strains were successfully obtained with high efficiency by expressing two guide RNA molecules from the genome-editing plasmid. Cotransformation of fungal cells with the genome-editing plasmid together with a circular donor DNA enabled marker-free multiplex gene deletion/integration in A. oryzae The presented repeatable marker-free genetic engineering approach for mutagenesis and gene deletion/integration will allow for efficient modification of multiple genes in industrial fungal strains, increasing their applicability.IMPORTANCE Multiple gene modifications of specific fungal strains are required for achieving industrial-scale production of enzymes and secondary metabolites. In the present study, we developed an efficient multiple genetic engineering technique for the filamentous fungus Aspergillus oryzae The approach is based on a clustered regulatory interspaced short palindromic repeats (CRISPR)/Cas9 system and recycling of an AMA1-based autonomous replicating plasmid. Because the plasmid harbors a drug resistance marker (ptrA), the approach does not require the construction of auxotrophic industrial strains prior to genome editing and allows for forced recycling of the gene-editing plasmid. The established plasmid-recycling technique involves an Aoace2-conditional expression cassette, whose induction severely impairs fungal growth. We used the developed genetic engineering techniques for highly efficient marker-free multiple gene deletion/integration in A. oryzae The genome-editing approaches established in the present study, which enable unlimited repeatable genetic engineering, will facilitate multiple gene modification of industrially important fungal strains.

Kishida, T., et al. (2016). "Specific Destruction of HIV Proviral p17 Gene in T Lymphoid Cells Achieved by the Genome Editing Technology." Front Microbiol **7**: 1001.

Recent development in genome editing technologies has enabled site-directed deprivation of a nucleotide sequence in the chromosome in mammalian cells. Human immunodeficiency (HIV) infection causes integration of proviral DNA into the chromosome, which potentially leads to re-emergence of the virus, but conventional treatment cannot delete the proviral DNA sequence from the cells infected with HIV. In the present study, the transcription activator-like effector nucleases (TALENs) specific for the HIV p17 gene were constructed, and their activities to destroy the target sequence were evaluated. SSA assay showed a high activity of a pair of p17-specific TALENs. A human T lymphoid cell line, Jurkat, was infected with a lentivirus vector followed by transfection with the TALEN-HIV by electroporation. The target sequence was destructed in approximately 10-95% of the p17 polymerase chain reaction clones, and the efficiencies depended on the Jurkat-HIV clones. Because p17 plays essential roles for assembly and budding of HIV, and this gene has relatively low nucleotide sequence diversity, genome editing procedures targeting p17 may provide a therapeutic benefit for HIV infection.

Korge, S., et al. (2015). "Highly Efficient Genome Editing via CRISPR/Cas9 to Create Clock Gene Knockout Cells." J Biol Rhythms **30**(5): 389-395.

Targeted genome editing using CRISPR/Cas9 is a relatively new, revolutionary technology allowing for efficient and directed alterations of the genome. It has been widely used for loss-of-function studies in animals and cell lines but has not yet been used to study circadian rhythms. Here, we describe the application of CRISPR/Cas9 genome editing for the generation of an F-box and leucine-rich repeat protein 3 (Fbxl3) knockout in a human cell line. Genomic alterations at the Fbxl3 locus occurred with very high efficiency (70%-100%) and specificity at both alleles, resulting in insertions and deletions that led to premature stop codons and hence FBXL3 knockout. Fbxl3 knockout cells displayed low amplitude and long period oscillations of Bmal1-luciferase reporter activity as well as increased CRY1 protein stability in line with previously published phenotypes for Fbxl3 knockout in mice. Thus, CRISPR/Cas9 genome editing should be highly valuable for studying circadian rhythms not only in human cells but also in classic model systems as well as nonmodel organisms.

Kunzelmann, S. and K. Forstemann (2017). "Reversible perturbations of gene regulation after

genome editing in Drosophila cells." PLoS One **12**(6): e0180135.

The prokaryotic phage defense CRISPR/cas-system has developed into a versatile toolbox for genome engineering and genetic studies in many organisms. While many efforts were spent on analyzing the consequences of off-target effects, only few studies addressed side-effects that occur due to the targeted manipulation of the genome. Here, we show that the CRISPR/cas9-mediated integration of an epitope tag in combination with a selection cassette can trigger an siRNA-mediated, epigenetic genome surveillance pathway in Drosophila melanogaster cells. After homology-directed insertion of the sequence coding for the epitope tag and the selection marker, a moderate level of siRNAs covering the inserted sequence and extending into the targeted locus was detected. This response affected protein levels less than two-fold and it persisted even after single cell cloning. However, removal of the selection cassette abolished the siRNA generation, demonstrating that this response is reversible. Consistently, marker-free genome engineering did not trigger the same surveillance mechanism. These two observations indicate that the selection cassette we employed induces an aberrant transcriptional arrangement and ultimately sets off the siRNA production. There have been prior concerns about undesirable effects induced by selection markers, but fortunately we were able to show that at least one of the epigenetic changes reverts as the marker gene is excised. Although the effects observed were rather weak (less than twofold de-repression upon ago2 or dcr-2 knock-down), we recommend that when selection markers are used during genome editing, a strategy for their subsequent removal should always be included.

Li, C., et al. (2021). "CRISPR/Cas: a Nobel Prize award-winning precise genome editing technology for gene therapy and crop improvement." J Zhejiang Univ Sci B **22**(4): 253-284.

Since it was first recognized in bacteria and archaea as a mechanism for innate viral immunity in the early 2010s, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) has rapidly been developed into a robust, multifunctional genome editing tool with many uses. Following the discovery of the initial CRISPR/Cas-based system, the technology has been advanced to facilitate a multitude of different functions. These include development as a base editor, prime editor, epigenetic editor, and CRISPR interference (CRISPRi) and CRISPR activator (CRISPRa) gene regulators. It can also be used for chromatin and RNA targeting and imaging. Its applications have proved revolutionary across numerous biological fields, especially in biomedical and agricultural improvement. As a diagnostic tool, CRISPR has been developed to aid the detection and screening of both human and plant diseases, and has even been applied during the current coronavirus disease 2019 (COVID-19) pandemic. CRISPR/Cas is also being trialed as a new form of gene therapy for treating various human diseases, including cancers, and has aided drug development. In terms of agricultural breeding, precise targeting of biological pathways via CRISPR/Cas has been key to regulating molecular biosynthesis and allowing modification of proteins, starch, oil, and other functional components for crop improvement. Adding to this, CRISPR/Cas has been shown capable of significantly enhancing both plant tolerance to environmental stresses and overall crop yield via the targeting of various agronomically important gene regulators. Looking to the future, increasing the efficiency and precision of CRISPR/Cas delivery systems and limiting off-target activity are two major challenges for wider application of the technology. This review provides an in-depth overview of current CRISPR development, including the advantages and disadvantages of the technology, recent applications, and future considerations.

Li, C. and H. Q. Lou (2015). "From gene editing to genome reconstitution: evolving techniques in yeast." Yi Chuan **37**(10): 1021-1028.

Homologous recombination is one of the main repair pathways in response to DNA double strand break (DSB) in eukaryotes. Based on this, a series of techniques to introduce DSB have been developed in order to edit the DNA sequence of genome. In eukaryotes, the gene editing technique was first established in S. cerevisiae by transformation of a foreign DNA fragment containing the sequence homologous to the targeted site more than thirty years ago. The core of all currently available editing methods lies in the introduction of DSB. Here, we try to convey a historic view of various editing techniques from its original version to the up-to-dated genome synthesis and reconstitution. We believe that this review will help to illustrate the trend of the development of genome editing techniques, which will provide a valuable reference for developing similar techniques in mammals.

Li, M., et al. (2019). "One-Step Generation of Seamless Luciferase Gene Knockin Using CRISPR/Cas9 Genome Editing in Human Pluripotent Stem Cells." Methods Mol Biol **1942**: 61-69.

Human pluripotent stem cells (hPSCs) offer powerful platforms for studying mechanisms of human diseases and for evaluating potential treatments. Genome editing, particularly the CRISPR/Cas9-based method, is highly effective for generating cell and

animal models to study genetic human diseases. However, the procedure for generating gene-edited hPSCs is laborious, time consuming and unintentional genetic changes may confound the consequent experiments and conclusions. Here we describe one-step knockin of the NanoLuc luciferase gene (Nluc) to the fragile X syndrome gene, FMR1, in a human embryonic stem cell line (hESC), H1, and a fragile X disease model human induced pluripotent stem cell line (hiPSC), FX-iPSC. The luciferase reporter cell lines provide new platforms for exploring potential treatments for fragile X syndrome. The shortened and scarless targeting method described here can be effectively applied to other genes.

Li, Q., et al. (2019). "Applications of Genome Editing Technology in Animal Disease Modeling and Gene Therapy." Comput Struct Biotechnol J **17**: 689-698.

Genome editing technology is a technique for targeted genetic modifications, enabling the knockout and addition of specific DNA fragments. This technology has been widely used in various types of biomedical research, clinics and agriculture. In terms of disease research, constructing appropriate animal models is necessary. Combining reproductive technology with genome editing, many animal disease models have been generated for basic and clinical research. In addition, precisely targeted modifications allow genome editing to flourish in the field of gene therapy. Many mutations refractory to traditional gene therapy could be permanently corrected at the DNA level. Thus, genome editing is undoubtedly a promising technology for gene therapy. In this review, we mainly introduce the applications of genome editing in constructing animal disease models and gene therapies, as well as its future prospects and challenges.

Li, T., et al. (2019). "CRISPR-Cpf1-mediated genome editing and gene regulation in human cells." Biotechnol Adv **37**(1): 21-27.

Clustered regularly interspaced short palindromic repeat (CRISPR) system is being championed as a robust and flexible tool for genome editing. Compared with CRISPR associated protein 9 (Cas9), the CRISPR from Prevotella and Francisella 1 (Cpf1) protein has some distinct characteristics, including RNase activity, T-rich protospacer adjacent motif (PAM) preference and generation of sticky cutting ends. The extremely low propensity of off-target effects and relatively high editing efficiency represent prominent advantages of Cpf1 over Cas9. CRISPR-Cpf1, alone or fused with function domains, has broadly expanded the applications such as multiplex gene knockout, transcriptional repression or activation and epigenome editing in a drug controlled way. Meanwhile, the modification of CRISPR RNAs (crRNAs) with aptamer RNA achieves great promotion on genome editing. Moreover, disease-associated gene manipulation in mice, tumor mutation detection in patients with cancers, and more yet to come, represent growing demands of CRISPR-Cpf1 in clinical genome therapy. In this review, we summarized the unique properties of Cpf1 and the molecular mechanisms underlying CRISPR-Cpf1 on gene editing and regulation in human cells.

Li, X., et al. (2015). "Outbred genome sequencing and CRISPR/Cas9 gene editing in butterflies." Nat Commun **6**: 8212.

Butterflies are exceptionally diverse but their potential as an experimental system has been limited by the difficulty of deciphering heterozygous genomes and a lack of genetic manipulation technology. Here we use a hybrid assembly approach to construct high-quality reference genomes for Papilio xuthus (contig and scaffold N50: 492 kb, 3.4 Mb) and Papilio machaon (contig and scaffold N50: 81 kb, 1.15 Mb), highly heterozygous species that differ in host plant affiliations, and adult and larval colour patterns. Integrating comparative genomics and analyses of gene expression yields multiple insights into butterfly evolution, including potential roles of specific genes in recent diversification. To functionally test gene function, we develop an efficient (up to 92.5%) CRISPR/Cas9 gene editing method that yields obvious phenotypes with three genes, Abdominal-B, ebony and frizzled. Our results provide valuable genomic and technological resources for butterflies and unlock their potential as a genetic model system.

Liu, B. Y., et al. (2018). "Tumor targeted genome editing mediated by a multi-functional gene vector for regulating cell behaviors." J Control Release **291**: 90-98.

For effective regulation of cell behaviors and prevention of tumor development by genome editing, we constructed multi-functional self-assembled nanoparticles based on natural polymers to deliver CRISPR-Cas9 plasmid to tumorous cells. The CRISPR based gene editing plasmid to knockout CDK11 gene was complexed with protamine sulfate, and then the complex was decorated by a multi-functional outer layer composed of an endosomolytic peptide (KALA) and aptamer AS1411 incorporated carboxymethyl chitosan. The resultant multi-functional nanoparticles, which exhibit significantly enhanced delivery efficiency, can specifically deliver the plasmid into tumor cell nuclei owing to the favorable effects of KALA in cellular uptake and endosomal escape, together with the cancer cell and cell nucleus targeting capability of AS1411 ligands. The genome editing mediated by the nanoparticles leads to a dramatic

decrease (>75%) in CDK11 expression, which results in further modulation of cancer cells with significant down-regulation of the proteins (MMP-9 and VEGF) involved in tumor development and metastasis as well as up-regulation of the tumor suppressor protein p53. More importantly, the detection of immune-related proteins after genome editing shows that the significantly enhanced Fas, CD80, MICA, MICB, and HLA-1 expression and decreased CD47 and MUC1 expression, indicating the genome editing is favorable for reversal of tumor-induced immunosuppression and prevention of tumor development.

Liu, G., et al. (2021). "Genome-wide identification and gene-editing of pigment transporter genes in the swallowtail butterfly Papilio xuthus." BMC Genomics **22**(1): 120.

BACKGROUND: Insect body coloration often functions as camouflage to survive from predators or mate selection. Transportation of pigment precursors or related metabolites from cytoplasm to subcellular pigment granules is one of the key steps in insect pigmentation and usually executed via such transporter proteins as the ATP-binding cassette (ABC) transmembrane transporters and small G-proteins (e.g. Rab protein). However, little is known about the copy numbers of pigment transporter genes in the butterfly genomes and about the roles of pigment transporters in the development of swallowtail butterflies. RESULTS: Here, we have identified 56 ABC transporters and 58 Rab members in the genome of swallowtail butterfly Papilio xuthus. This is the first case of genome-wide gene copy number identification of ABC transporters in swallowtail butterflies and Rab family in lepidopteran insects. Aiming to investigate the contribution of the five genes which are orthologous to well-studied pigment transporters (ABCG: white, scarlet, brown and ok; Rab: lightoid) of fruit fly or silkworm during the development of swallowtail butterflies, we performed CRISPR/Cas9 gene-editing of these genes using P. xuthus as a model and sequenced the transcriptomes of their morphological mutants. Our results indicate that the disruption of each gene produced mutated phenotypes in the colors of larvae (cuticle, testis) and/or adult eyes in G0 individuals but have no effect on wing color. The transcriptomic data demonstrated that mutations induced by CRISPR/Cas9 can lead to the accumulation of abnormal transcripts and the decrease or dosage compensation of normal transcripts at gene expression level. Comparative transcriptomes revealed 606 ~ 772 differentially expressed genes (DEGs) in the mutants of four ABCG transporters and 1443 DEGs in the mutants of lightoid. GO and KEGG enrichment analysis showed that DEGs in ABCG transporter mutants enriched to the oxidoreductase activity, heme binding, iron ion binding process possibly related to the color display, and DEGs in lightoid mutants are enriched in glycoprotein binding and protein kinases. CONCLUSIONS: Our data indicated these transporter proteins play an important role in body color of P. xuthus. Our study provides new insights into the function of ABC transporters and small G-proteins in the morphological development of butterflies.

Liu, H., et al. (2016). "Genome Editing of Wnt-1, a Gene Associated with Segmentation, via CRISPR/Cas9 in the Pine Caterpillar Moth, Dendrolimus punctatus." Front Physiol **7**: 666.

The pine caterpillar moth, Dendrolimus punctatus, is a devastating forest pest. Genetic manipulation of this insect pest is limited due to the lack of genomic and functional genomic toolsets. Recently, CRISPR/Cas9 technology has been demonstrated to be a promising approach to modify the genome. To investigate gene functions during the embryogenesis, we introduced CRISPR/Cas9 system in D. punctatus to precisely and effectively manipulate gene expressions inmutant embryos. Compared to controls, knocking out of DpWnt-1, a gene well known for its role in the early body planning, led to high embryonic mortality. Among these mutants, 32.9% of the embryos and larvae showed an abnormal development. DpWnt-1 mutants predominantly exhibited abnormal posterior segments. In addition, multiple phenotypes were observed, including the loss of limbs and the head deformation, suggesting that DpWnt-1 signaling pathway is necessary for anterior segmentation and appendage development. Overall, our results demonstrate that CRISPR/Cas9 system is feasible and efficient in inducing mutations at a specific locus in D. punctatus. This study not only lays the foundation for characterizing gene functions in a non-model species, but also facilitates the future development of pest control alternatives for a major defoliator.

Liu, Y., et al. (2018). "High GC Content Cas9-Mediated Genome-Editing and Biosynthetic Gene Cluster Activation in Saccharopolyspora erythraea." ACS Synth Biol **7**(5): 1338-1348.

The overexpression of bacterial secondary metabolite biosynthetic enzymes is the basis for industrial overproducing strains. Genome editing tools can be used to further improve gene expression and yield. Saccharopolyspora erythraea produces erythromycin, which has extensive clinical applications. In this study, the CRISPR-Cas9 system was used to edit genes in the S. erythraea genome. A temperature-sensitive plasmid containing the PermE promoter, to drive Cas9 expression, and the Pj23119 and PkasO promoters, to drive sgRNAs, was designed.

Erythromycin esterase, encoded by S. erythraea SACE_1765, inactivates erythromycin by hydrolyzing the macrolactone ring. Sequencing and qRT-PCR confirmed that reporter genes were successfully inserted into the SACE_1765 gene. Deletion of SACE_1765 in a high-producing strain resulted in a 12.7% increase in erythromycin levels. Subsequent PermE- egfp knock-in at the SACE_0712 locus resulted in an 80.3% increase in erythromycin production compared with that of wild type. Further investigation showed that PermE promoter knock-in activated the erythromycin biosynthetic gene clusters at the SACE_0712 locus. Additionally, deletion of indA (SACE_1229) using dual sgRNA targeting without markers increased the editing efficiency to 65%. In summary, we have successfully applied Cas9-based genome editing to a bacterial strain, S. erythraea, with a high GC content. This system has potential application for both genome-editing and biosynthetic gene cluster activation in Actinobacteria.

Lo Scrudato, M., et al. (2019). "Genome Editing of Expanded CTG Repeats within the Human DMPK Gene Reduces Nuclear RNA Foci in the Muscle of DM1 Mice." Mol Ther **27**(8): 1372-1388.

Myotonic dystrophy type 1 (DM1) is caused by a CTG repeat expansion located in the 3' UTR of the DMPK gene. Expanded DMPK transcripts aggregate into nuclear foci and alter the function of RNA-binding proteins, leading to defects in the alternative splicing of numerous pre-mRNAs. To date, there is no curative treatment for DM1. Here we investigated a gene-editing strategy using the CRISPR-Cas9 system from Staphylococcus aureus (Sa) to delete the CTG repeats in the human DMPK locus. Co-expression of SaCas9 and selected pairs of single-guide RNAs (sgRNAs) in cultured DM1 patient-derived muscle line cells carrying 2,600 CTG repeats resulted in targeted DNA deletion, ribonucleoprotein foci disappearance, and correction of splicing abnormalities in various transcripts. Furthermore, a single intramuscular injection of recombinant AAV vectors expressing CRISPR-SaCas9 components in the tibialis anterior muscle of DMSXL (myotonic dystrophy mouse line carrying the human DMPK gene with >1,000 CTG repeats) mice decreased the number of pathological RNA foci in myonuclei. These results establish the proof of concept that genome editing of a large trinucleotide expansion is feasible in muscle and may represent a useful strategy to be further developed for the treatment of myotonic dystrophy.

Ma, S., et al. (2014). "Genome editing of BmFib-H gene provides an empty Bombyx mori silk gland for a highly efficient bioreactor." Sci Rep **4**: 6867.

Evolution has produced some remarkable creatures, of which silk gland is a fascinating organ that exists in a variety of insects and almost half of the 34,000 spider species. The impressive ability to secrete huge amount of pure silk protein, and to store proteins at an extremely high concentration (up to 25%) make the silk gland of Bombyx mori hold great promise to be a cost-effective platform for production of recombinant proteins. However, the extremely low production yields of the numerous reported expression systems greatly hindered the exploration and application of silk gland bioreactors. Using customized zinc finger nucleases (ZFN), we successfully performed genome editing of Bmfib-H gene, which encodes the largest and most abundant silk protein, in B. mori with efficiency higher than any previously reported. The resulted Bmfib-H knocked-out B. mori showed a smaller and empty silk gland, abnormally developed posterior silk gland cells, an extremely thin cocoon that contain only sericin proteins, and a slightly heavier pupae. We also showed that removal of endogenous Bmfib-H protein could significantly increase the expression level of exogenous protein. Furthermore, we demonstrated that the bioreactor is suitable for large scale production of protein-based materials.

Maeder, M. L. and C. A. Gersbach (2016). "Genome-editing Technologies for Gene and Cell Therapy." Mol Ther **24**(3): 430-446.

Gene therapy has historically been defined as the addition of new genes to human cells. However, the recent advent of genome-editing technologies has enabled a new paradigm in which the sequence of the human genome can be precisely manipulated to achieve a therapeutic effect. This includes the correction of mutations that cause disease, the addition of therapeutic genes to specific sites in the genome, and the removal of deleterious genes or genome sequences. This review presents the mechanisms of different genome-editing strategies and describes each of the common nuclease-based platforms, including zinc finger nucleases, transcription activator-like effector nucleases (TALENs), meganucleases, and the CRISPR/Cas9 system. We then summarize the progress made in applying genome editing to various areas of gene and cell therapy, including antiviral strategies, immunotherapies, and the treatment of monogenic hereditary disorders. The current challenges and future prospects for genome editing as a transformative technology for gene and cell therapy are also discussed.

Maier, R. M., et al. (1995). "Complete sequence of the maize chloroplast genome: gene content, hotspots of divergence and fine tuning of genetic information by transcript editing." J Mol Biol **251**(5): 614-628.

The nucleotide sequence of the chloroplast (cp) DNA from maize (Zea mays) has been completed. The circular double-stranded DNA, which consists of 140,387 base-pairs, contains a pair of inverted repeat regions (IRA and IRB) with 22,748 base-pairs each, which are separated by a small and a large single copy region (SSC and LSC) of 12,536 and 82,355 base-pairs, respectively. The gene content and the relative positions of a total of 104 genes (70 peptide-encoding genes, 30 tRNA genes and four rRNA genes) are identical with the chloroplast DNA of the closely related species rice (Oryza sativa). A detailed analysis of the two graminean plastomes allows the identification of hotspots of divergence which predominate in one region containing a cluster of tRNA genes and in two regions containing degenerated reading frames. One of these length differences is thought to reflect a gene transfer event from the plastome to the nucleus, which is followed by progressive degradation of the respective chloroplast gene resulting in gene fragments. The other divergent plastome region seems to be due to the complete loss of a plastid gene and its functional substitution by a nuclear encoded eukaryotic homologue. The rate of neutral nucleotide substitutions is significantly reduced for protein coding genes located in the inverted repeat regions. This indicates that the existence of inverted repeat regions confers increased genetic stability of the genes positioned in these regions as compared to genes located in the two single copy regions. Editing events cause the primary structures of several transcripts to deviate from the corresponding genomic sequences by C to U transitions. The unambiguous deduction of amino acid sequences from the nucleotide sequences of the corresponding genes is, therefore, not possible. A survey of the 25 editing positions identified in 13 different transcripts of the maize plastome shows that representatives of all protein coding gene classes are subject to editing. A strong bias exists for the second codon position and for certain codon transitions. Based on the number and the codon transition types, and taking into account the frequency of putative editing sites in all peptide encoding genes and unidentified reading frames, a total number of only few more than the experimentally verified 25 editing sites encoded in the maize plastome is estimated. This corresponds to 0.13% of amino acid positions which cannot be derived from the corresponding codons present in the corresponding genes.

Makai, S., et al. (2016). "A Catalog of Regulatory Sequences for Trait Gene for the Genome Editing of Wheat." Front Plant Sci **7**: 1504.

Wheat has been cultivated for 10000 years and ever since the origin of hexaploid wheat it has been exempt from natural selection. Instead, it was under the constant selective pressure of human agriculture from harvest to sowing during every year, producing a vast array of varieties. Wheat has been adopted globally, accumulating variation for genes involved in yield traits, environmental adaptation and resistance. However, one small but important part of the wheat genome has hardly changed: the regulatory regions of both the x- and y-type high molecular weight glutenin subunit (HMW-GS) genes, which are alone responsible for approximately 12% of the grain protein content. The phylogeny of the HMW-GS regulatory regions of the Triticeae demonstrates that a genetic bottleneck may have led to its decreased diversity during domestication and the subsequent cultivation. It has also highlighted the fact that the wild relatives of wheat may offer an unexploited genetic resource for the regulatory region of these genes. Significant research efforts have been made in the public sector and by international agencies, using wild crosses to exploit the available genetic variation, and as a result synthetic hexaploids are now being utilized by a number of breeding companies. However, a newly emerging tool of genome editing provides significantly improved efficiency in exploiting the natural variation in HMW-GS genes and incorporating this into elite cultivars and breeding lines. Recent advancement in the understanding of the regulation of these genes underlines the needs for an overview of the regulatory elements for genome editing purposes.

Mann, C. M., et al. (2019). "The Gene Sculpt Suite: a set of tools for genome editing." Nucleic Acids Res **47**(W1): W175-W182.

The discovery and development of DNA-editing nucleases (Zinc Finger Nucleases, TALENs, CRISPR/Cas systems) has given scientists the ability to precisely engineer or edit genomes as never before. Several different platforms, protocols and vectors for precision genome editing are now available, leading to the development of supporting web-based software. Here we present the Gene Sculpt Suite (GSS), which comprises three tools: (i) GTagHD, which automatically designs and generates oligonucleotides for use with the GeneWeld knock-in protocol; (ii) MEDJED, a machine learning method, which predicts the extent to which a double-stranded DNA break site will utilize the microhomology-mediated repair pathway; and (iii) MENTHU, a tool for identifying genomic locations likely to give rise to a single predominant microhomology-mediated end joining allele (PreMA) repair outcome. All tools in the GSS are freely available for download under the GPL v3.0 license and can be run locally on Windows, Mac and Linux systems capable of running R and/or Docker. The GSS is also freely available online at www.genesculpt.org.

Martinez, V., et al. (2017). "CRISPR/Cas9-based genome editing for simultaneous interference with gene expression and protein stability." Nucleic Acids Res **45**(20): e171.

Interference with genes is the foundation of reverse genetics and is key to manipulation of living cells for biomedical and biotechnological applications. However, classical genetic knockout and transcriptional knockdown technologies have different drawbacks and offer no control over existing protein levels. Here, we describe an efficient genome editing approach that affects specific protein abundances by changing the rates of both RNA synthesis and protein degradation, based on the two cross-kingdom control mechanisms CRISPRi and the N-end rule for protein stability. In addition, our approach demonstrates that CRISPRi efficiency is dependent on endogenous gene expression levels. The method has broad applications in e.g. study of essential genes and antibiotics discovery.

Maule, G., et al. (2020). "Gene Therapy for Cystic Fibrosis: Progress and Challenges of Genome Editing." Int J Mol Sci **21**(11).

Since the early days of its conceptualization and application, human gene transfer held the promise of a permanent solution to genetic diseases including cystic fibrosis (CF). This field went through alternated periods of enthusiasm and distrust. The development of refined technologies allowing site specific modification with programmable nucleases highly revived the gene therapy field. CRISPR nucleases and derived technologies tremendously facilitate genome manipulation offering diversified strategies to reverse mutations. Here we discuss the advancement of gene therapy, from therapeutic nucleic acids to genome editing techniques, designed to reverse genetic defects in CF. We provide a roadmap through technologies and strategies tailored to correct different types of mutations in the cystic fibrosis transmembrane regulator (CFTR) gene, and their applications for the development of experimental models valuable for the advancement of CF therapies.

Meissner, T. B., et al. (2014). "Genome editing for human gene therapy." Methods Enzymol **546**: 273-295.

The rapid advancement of genome-editing techniques holds much promise for the field of human gene therapy. From bacteria to model organisms and human cells, genome editing tools such as zinc-finger nucleases (ZNFs), TALENs, and CRISPR/Cas9 have been successfully used to manipulate the respective genomes with unprecedented precision. With regard to human gene therapy, it is of great interest to test the feasibility of genome editing in primary human hematopoietic cells that could potentially be used to treat a variety of human genetic disorders such as hemoglobinopathies, primary immunodeficiencies, and cancer. In this chapter, we explore the use of the CRISPR/Cas9 system for the efficient ablation of genes in two clinically relevant primary human cell types, CD4+ T cells and CD34+ hematopoietic stem and progenitor cells. By using two guide RNAs directed at a single locus, we achieve highly efficient and predictable deletions that ablate gene function. The use of a Cas9-2A-GFP fusion protein allows FACS-based enrichment of the transfected cells. The ease of designing, constructing, and testing guide RNAs makes this dual guide strategy an attractive approach for the efficient deletion of clinically relevant genes in primary human hematopoietic stem and effector cells and enables the use of CRISPR/Cas9 for gene therapy.

Mesuraca, M., et al. (2018). "Turning Stem Cells Bad: Generation of Clinically Relevant Models of Human Acute Myeloid Leukemia through Gene Delivery- or Genome Editing-Based Approaches." Molecules **23**(8).

Acute myeloid leukemia (AML), the most common acute leukemia in the adult, is believed to arise as a consequence of multiple molecular events that confer on primitive hematopoietic progenitors unlimited self-renewal potential and cause defective differentiation. A number of genetic aberrations, among which a variety of gene fusions, have been implicated in the development of a transformed phenotype through the generation of dysfunctional molecules that disrupt key regulatory mechanisms controlling survival, proliferation, and differentiation in normal stem and progenitor cells. Such genetic aberrations can be recreated experimentally to a large extent, to render normal hematopoietic stem cells "bad", analogous to the leukemic stem cells. Here, we wish to provide a brief outline of the complementary experimental approaches, largely based on gene delivery and more recently on gene editing, employed over the last two decades to gain insights into the molecular mechanisms underlying AML development and progression and on the prospects that their applications offer for the discovery and validation of innovative therapies.

Milani, M., et al. (2017). "Genome editing for scalable production of alloantigen-free lentiviral vectors for in vivo gene therapy." EMBO Mol Med **9**(11): 1558-1573.

Lentiviral vectors (LV) are powerful and versatile vehicles for gene therapy. However, their complex biological composition challenges large-scale manufacturing and raises concerns for in vivo applications, because particle components and contaminants may trigger immune responses. Here, we

show that producer cell-derived polymorphic class-I major histocompatibility complexes (MHC-I) are incorporated into the LV surface and trigger allogeneic T-cell responses. By disrupting the beta-2 microglobulin gene in producer cells, we obtained MHC-free LV with substantially reduced immunogenicity. We introduce this targeted editing into a novel stable LV packaging cell line, carrying single-copy inducible vector components, which can be reproducibly converted into high-yield LV producers upon site-specific integration of the LV genome of interest. These LV efficiently transfer genes into relevant targets and are more resistant to complement-mediated inactivation, because of reduced content of the vesicular stomatitis virus envelope glycoprotein G compared to vectors produced by transient transfection. Altogether, these advances support scalable manufacturing of alloantigen-free LV with higher purity and increased complement resistance that are better suited for in vivo gene therapy.

Miyaoka, Y., et al. (2018). "Detection and Quantification of HDR and NHEJ Induced by Genome Editing at Endogenous Gene Loci Using Droplet Digital PCR." Methods Mol Biol **1768**: 349-362.

Genome editing holds great promise for experimental biology and potential clinical use. To successfully utilize genome editing, it is critical to sensitively detect and quantify its outcomes: homology-directed repair (HDR) and nonhomologous end joining (NHEJ). This has been difficult at endogenous gene loci and instead is frequently done using artificial reporter systems. Here, we describe a droplet digital PCR (ddPCR)-based method to simultaneously measure HDR and NHEJ at endogenous gene loci. This highly sensitive and quantitative method may significantly contribute to a better understanding of DNA repair mechanisms underlying genome editing and to the improvement of genome editing technology by allowing for efficient and systematic testing of many genome editing conditions in parallel.

Mizuno, N., et al. (2018). "Intra-embryo Gene Cassette Knockin by CRISPR/Cas9-Mediated Genome Editing with Adeno-Associated Viral Vector." iScience **9**: 286-297.

Intra-embryo genome editing by CRISPR/Cas9 enables easy generation of gene-modified animals by non-homologous end joining (NHEJ)-mediated frameshift mutations or homology-directed repair (HDR)-mediated point mutations. However, large modifications, such as gene replacement or gene fusions, are still difficult to introduce in embryos without costly micromanipulators. Moreover, micromanipulation techniques for intra-embryo genome editing have been established in only a small set of animals. To overcome these issues, we developed a method of large-fragment DNA knockin without micromanipulation. In this study, we successfully delivered the knockin donor DNA into zygotes by adeno-associated virus (AAV) without removing the zona pellucida, and we succeeded in both large-DNA fragment knockin and whole exon exchange with electroporation of CRISPR/Cas9 ribonucleoprotein. By this method, we can exchange large DNA fragments conveniently in various animal species without micromanipulation.

Mizuno-Iijima, S., et al. (2020). "Efficient production of large deletion and gene fragment knock-in mice mediated by genome editing with Cas9-mouse Cdt1 in mouse zygotes." Methods.

Genetically modified mouse models are essential for in vivo investigation of gene function and human disease research. Targeted mutations can be introduced into mouse embryos using genome editing technology such as CRISPR-Cas. Although mice with small indel mutations can be produced, the production of mice carrying large deletions or gene fragment knock-in alleles remains inefficient. We introduced the nuclear localisation property of Cdt1 protein into the CRISPR-Cas system for efficient production of genetically engineered mice. Mouse Cdt1-connected Cas9 (Cas9-mC) was present in the nucleus of HEK293T cells and mouse embryos. Cas9-mC induced a bi-allelic full deletion of Dmd, GC-rich fragment knock-in, and floxed allele knock-in with high efficiency compared to standard Cas9. These results indicate that Cas9-mC is a useful tool for producing mouse models carrying targeted mutations.

Morales, D. P., et al. (2018). "Light-Triggered Genome Editing: Cre Recombinase Mediated Gene Editing with Near-Infrared Light." Small **14**(30): e1800543.

A light-activated genome editing platform based on the release of enzymes from a plasmonic nanoparticle carrier when exposed to biocompatible near-infrared light pulses is described. The platform relies on the robust affinity of polyhistidine tags to nitrilotriacetic acid in the presence of copper which is attached to double-stranded nucleic acids self-assembled on the gold nanoparticle surface. A protein fusion of the Cre recombinase containing a TAT internalization peptide sequence to achieve endosomal localization is also employed. High-resolution gene knock-in of a red fluorescent reporter is observed using a commercial two-photon microscope. High-throughput irradiation is described to generate useful quantities of edited cells.

Morineau, C., et al. (2017). "Selective gene dosage by CRISPR-Cas9 genome editing in hexaploid Camelina sativa." Plant Biotechnol J **15**(6): 729-739.

In many plant species, gene dosage is an important cause of phenotype variation. Engineering gene dosage, particularly in polyploid genomes, would provide an efficient tool for plant breeding. The hexaploid oilseed crop Camelina sativa, which has three closely related expressed subgenomes, is an ideal species for investigation of the possibility of creating a large collection of combinatorial mutants. Selective, targeted mutagenesis of the three delta-12-desaturase (FAD2) genes was achieved by CRISPR-Cas9 gene editing, leading to reduced levels of polyunsaturated fatty acids and increased accumulation of oleic acid in the oil. Analysis of mutations over four generations demonstrated the presence of a large variety of heritable mutations in the three isologous CsFAD2 genes. The different combinations of single, double and triple mutants in the T3 generation were isolated, and the complete loss-of-function mutants revealed the importance of delta-12-desaturation for Camelina development. Combinatorial association of different alleles for the three FAD2 loci provided a large diversity of Camelina lines with various lipid profiles, ranging from 10% to 62% oleic acid accumulation in the oil. The different allelic combinations allowed an unbiased analysis of gene dosage and function in this hexaploid species, but also provided a unique source of genetic variability for plant breeding.

Morita, H., et al. (2017). "Exogenous gene integration mediated by genome editing technologies in zebrafish." Bioengineered **8**(3): 287-295.

Genome editing technologies, such as transcription activator-like effector nuclease (TALEN) and the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein (Cas) systems, can induce DNA double-strand breaks (DSBs) at the targeted genomic locus, leading to frameshift-mediated gene disruption in the process of DSB repair. Recently, the technology-induced DSBs followed by DSB repairs are applied to integrate exogenous genes into the targeted genomic locus in various model organisms. In addition to a conventional knock-in technology mediated by homology-directed repair (HDR), novel knock-in technologies using refined donor vectors have also been developed with the genome editing technologies based on other DSB repair mechanisms, including non-homologous end joining (NHEJ) and microhomology-mediated end joining (MMEJ). Therefore, the improved knock-in technologies would contribute to freely modify the genome of model organisms.

Mungpakdee, S., et al. (2014). "Massive gene transfer and extensive RNA editing of a symbiotic dinoflagellate plastid genome." Genome Biol Evol **6**(6): 1408-1422.

Genome sequencing of Symbiodinium minutum revealed that 95 of 109 plastid-associated genes have been transferred to the nuclear genome and subsequently expanded by gene duplication. Only 14 genes remain in plastids and occur as DNA minicircles. Each minicircle (1.8-3.3 kb) contains one gene and a conserved noncoding region containing putative promoters and RNA-binding sites. Nine types of RNA editing, including a novel G/U type, were discovered in minicircle transcripts but not in genes transferred to the nucleus. In contrast to DNA editing sites in dinoflagellate mitochondria, which tend to be highly conserved across all taxa, editing sites employed in DNA minicircles are highly variable from species to species. Editing is crucial for core photosystem protein function. It restores evolutionarily conserved amino acids and increases peptidyl hydropathy. It also increases protein plasticity necessary to initiate photosystem complex assembly.

Muth, G. (2018). "The pSG5-based thermosensitive vector family for genome editing and gene expression in actinomycetes." Appl Microbiol Biotechnol **102**(21): 9067-9080.

Actinomycetes are the most important producers of secondary metabolites for medical, agricultural and industrial applications. Efficient engineering of bacterial genomes to improve their biosynthetic capabilities largely depends on the available arsenal of tools and vectors. One of the most widely used vector systems for actinomycetes is derived from the Streptomyces ghanaensis DSM2932 plasmid pSG5. pSG5 is a broad host range multicopy plasmid replicating via a rolling circle mechanism. The unique feature of pSG5, which distinguishes it from other Streptomyces plasmids, is its naturally thermosensitive mode of replication. This allows the efficient elimination of the plasmid from its host by simply shifting the incubation temperature to non-permissive 37-39 degrees C. This property makes pSG5 derivatives ideal facultative suicide vectors required for selection of gene disruption/gene replacement, transposon delivery or CRISPR/Cas9-mediated genome editing. Whereas these techniques depend on the fast elimination of the vector, stably replicating expression vectors for the production of recombinant proteins have been constructed more recently. This mini-review describes the generation and application of the pSG5 vector family, highlighting the specific features of the distinct vector plasmids.

Nakamura, H., et al. (2017). "Highly efficient gene targeting in Aspergillus oryzae industrial strains under ligD mutation introduced by genome editing: Strain-specific differences in the effects of deleting EcdR, the negative regulator of sclerotia formation." J Gen Appl Microbiol **63**(3): 172-178.

Numerous strains of Aspergillus oryzae are industrially used for Japanese traditional fermentation and for the production of enzymes and heterologous proteins. In A. oryzae, deletion of the ku70 or ligD genes involved in non-homologous end joining (NHEJ) has allowed high gene targeting efficiency. However, this strategy has been mainly applied under the genetic background of the A. oryzae wild strain RIB40, and it would be laborious to delete the NHEJ genes in many A. oryzae industrial strains, probably due to their low gene targeting efficiency. In the present study, we generated ligD mutants from the A. oryzae industrial strains by employing the CRISPR/Cas9 system, which we previously developed as a genome editing method. Uridine/uracil auxotrophic strains were generated by deletion of the pyrG gene, which was subsequently used as a selective marker. We examined the gene targeting efficiency with the ecdR gene, of which deletion was reported to induce sclerotia formation under the genetic background of the strain RIB40. As expected, the deletion efficiencies were high, around 60~80%, in the ligD mutants of industrial strains. Intriguingly, the effects of the ecdR deletion on sclerotia formation varied depending on the strains, and we found sclerotia-like structures under the background of the industrial strains, which have never been reported to form sclerotia. The present study demonstrates that introducing ligD mutation by genome editing is an effective method allowing high gene targeting efficiency in A. oryzae industrial strains.

Nakashima, N. and K. Miyazaki (2014). "Bacterial cellular engineering by genome editing and gene silencing." Int J Mol Sci **15**(2): 2773-2793.

Genome editing is an important technology for bacterial cellular engineering, which is commonly conducted by homologous recombination-based procedures, including gene knockout (disruption), knock-in (insertion), and allelic exchange. In addition, some new recombination-independent approaches have emerged that utilize catalytic RNAs, artificial nucleases, nucleic acid analogs, and peptide nucleic acids. Apart from these methods, which directly modify the genomic structure, an alternative approach is to conditionally modify the gene expression profile at the posttranscriptional level without altering the genomes. This is performed by expressing antisense RNAs to knock down (silence) target mRNAs in vivo. This review describes the features and recent advances on methods used in genomic engineering and silencing technologies that are advantageously used for bacterial cellular engineering.

Nakayasu, M., et al. (2018). "Generation of alpha-solanine-free hairy roots of potato by CRISPR/Cas9 mediated genome editing of the St16DOX gene." Plant Physiol Biochem **131**: 70-77.

Potato (Solanum tuberosum) is a major food crop, while the most tissues of potato accumulates steroidal glycoalkaloids (SGAs) alpha-solanine and alpha-chaconine. Since SGAs confer a bitter taste on human and show the toxicity against various organisms, reducing the SGA content in the tubers is requisite for potato breeding. However, generation of SGA-free potato has not been achieved yet, although silencing of several SGA biosynthetic genes led a decrease in SGAs. Here, we show that the knockout of St16DOX encoding a steroid 16alpha-hydroxylase in SGA biosynthesis causes the complete abolition of the SGA accumulation in potato hairy roots. Nine candidate guide RNA (gRNA) target sequences were selected from St16DOX by in silico analysis, and the two or three gRNAs were introduced into a CRISPR/Cas9 vector designated as pMgP237-2A-GFP that can express multiplex gRNAs based on the pre-tRNA processing system. To establish rapid screening of the candidate gRNAs that can efficiently mutate the St16DOX gene, we used a potato hairy root culture system for the introduction of the pMgP237 vectors. Among the transgenic hairy roots, two independent lines showed no detectable SGAs but accumulated the glycosides of 22,26-dihydroxycholesterol, which is the substrate of St16DOX. Analysis of the two lines with sequencing exhibited the mutated sequences of St16DOX with no wild-type sequences. Thus, generation of SGA-free hairy roots of tetraploid potato was achieved by the combination of the hairy root culture and the pMgP237-2A-GFP vector. This experimental system is useful to evaluate the efficacy of candidate gRNA target sequences in the short-term.

Namula, Z., et al. (2019). "Genome mutation after the introduction of the gene editing by electroporation of Cas9 protein (GEEP) system into bovine putative zygotes." In Vitro Cell Dev Biol Anim **55**(8): 598-603.

The present study was designed to investigate the effects of voltage strength on embryonic developmental rate and mutation efficiency in bovine putative zygotes during electroporation with the CRISPR/Cas9 system to target the MSTN gene at different time points after insemination. Results showed that there was no significant interaction between electroporation time and voltage strength on the embryonic cleavage and blastocyst formation rates. However, increasing the voltage strength to 20 V/mm to electroporate the zygotes at 10 h after the start of

insemination yielded significantly lower blastocyst formation rates ($P < 0.05$) than those of the 10-V/mm electroporated zygotes. Mutation efficiency was then assessed in individual blastocysts by DNA sequence analysis of the target sites in the MSTN gene. A positive correlation between mutation rate and voltage strength was observed. The mutation efficiency in mutant blastocysts was significantly higher in the zygotes electroporated with 20 V/mm at 10 h after the start of insemination ($P < 0.05$) than in the zygotes electroporated at 15 h, irrespective of the voltage strength. We also noted that a certain number of blastocysts from zygotes that were electroporated with more than 15 V/mm at 10 h (4.8-16.7%) and 20 V/mm at 15 h (4.8%) were biallelic mutants. Our results suggest that the voltage strength during electroporation as well as electroporation time certainly have effects on the embryonic developmental rate and mutation efficiency in bovine putative zygotes.

Nihongaki, Y., et al. (2019). "A split CRISPR-Cpf1 platform for inducible genome editing and gene activation." Nat Chem Biol **15**(9): 882-888.

The CRISPR-Cpf1 endonuclease has recently been demonstrated as a powerful tool to manipulate targeted gene sequences. Here, we performed an extensive screening of split Cpf1 fragments and identified a pair that, combined with inducible dimerization domains, enables chemical- and light-inducible genome editing in human cells. We also identified another split Cpf1 pair that is spontaneously activated. The newly generated amino and carboxyl termini of the spontaneously activated split Cpf1 can be repurposed as de novo fusion sites of artificial effector domains. Based on this finding, we generated an improved split dCpf1 activator, which has the potential to activate endogenous genes more efficiently than a previously established dCas9 activator. Finally, we showed that the split dCpf1 activator can efficiently activate target genes in mice. These results demonstrate that the present split Cpf1 provides an efficient and sophisticated genome manipulation in the fields of basic research and biotechnological applications.

Nishizawa-Yokoi, A., et al. (2015). "Precision genome editing in plants via gene targeting and piggyBac-mediated marker excision." Plant J **81**(1): 160-168.

Precise genome engineering via homologous recombination (HR)-mediated gene targeting (GT) has become an essential tool in molecular breeding as well as in basic plant science. As HR-mediated GT is an extremely rare event, positive-negative selection has been used extensively in flowering plants to isolate cells in which GT has occurred. In order to utilize GT as a methodology for precision mutagenesis, the positive selectable marker gene should be completely eliminated from the GT locus. Here, we introduce targeted point mutations conferring resistance to herbicide into the rice acetolactate synthase (ALS) gene via GT with subsequent marker excision by piggyBac transposition. Almost all regenerated plants expressing piggyBac transposase contained exclusively targeted point mutations without concomitant re-integration of the transposon, resulting in these progeny showing a herbicide bispyribac sodium (BS)-tolerant phenotype. This approach was also applied successfully to the editing of a microRNA targeting site in the rice cleistogamy 1 gene. Therefore, our approach provides a general strategy for the targeted modification of endogenous genes in plants.

Nishizawa-Yokoi, A., et al. (2016). "Seamless Genome Editing in Rice via Gene Targeting and Precise Marker Elimination." Methods Mol Biol **1469**: 137-146.

Positive-negative selection using hygromycin phosphotransferase (hpt) and diphtheria toxin A-fragment (DT-A) as positive and negative selection markers, respectively, allows enrichment of cells harboring target genes modified via gene targeting (GT). We have developed a successful GT system employing positive-negative selection and subsequent precise marker excision via the piggyBac transposon derived from the cabbage looper moth to introduce desired modifications into target genes in the rice genome. This approach could be applied to the precision genome editing of almost all endogenous genes throughout the genome, at least in rice.

Nunez-Munoz, L., et al. (2021). "Plant drought tolerance provided through genome editing of the trehalase gene." Plant Signal Behav **16**(4): 1877005.

Drought is one of the main abiotic factors that affect agricultural productivity, jeopardizing food security. Modern biotechnology is a useful tool for the generation of stress-tolerant crops, but its release and field-testing involves complex regulatory frameworks. However, gene editing technology mediated by the CRISPR/Cas9 system is a suitable strategy for plant breeding, which can lead to precise and specific modifications in the plant genome. The aim of the present work is to produce drought-tolerant plant varieties by modifying the trehalase gene. Furthermore, a new vector platform was developed to edit monocot and dicot genomes, by modifying vectors adding a streptomycin resistance marker for use with the hypervirulent Agrobacterium tumefaciens AGL1 strain. The gRNA design was based on the trehalase sequence in several species of the genus Selaginella that show drought tolerance. Arabidopsis thaliana carrying editions in the trehalase substrate-binding domain showed a higher tolerance to drought stress. In

addition, a transient transformation system for gene editing in maize leaves was characterized.

Oh, H. S., et al. (2019). "Herpesviral lytic gene functions render the viral genome susceptible to novel editing by CRISPR/Cas9." Elife **8**.

Herpes simplex virus (HSV) establishes lifelong latent infection and can cause serious human disease, but current antiviral therapies target lytic but not latent infection. We screened for sgRNAs that cleave HSV-1 DNA sequences efficiently in vitro and used these sgRNAs to observe the first editing of quiescent HSV-1 DNA. The sgRNAs targeted lytic replicating viral DNA genomes more efficiently than quiescent genomes, consistent with the open structure of lytic chromatin. Editing of latent genomes caused short indels while editing of replicating genomes produced indels, linear molecules, and large genomic sequence loss around the gRNA target site. The HSV ICP0 protein and viral DNA replication increased the loss of DNA sequences around the gRNA target site. We conclude that HSV, by promoting open chromatin needed for viral gene expression and by inhibiting the DNA damage response, makes the genome vulnerable to a novel form of editing by CRISPR-Cas9 during lytic replication.

Okuzaki, A., et al. (2018). "CRISPR/Cas9-mediated genome editing of the fatty acid desaturase 2 gene in Brassica napus." Plant Physiol Biochem **131**: 63-69.

The CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9-mediated genome editing system has been widely applied as a powerful tool for modifying preferable endogenous genes. This system is highly expected to be further applied for the breeding of various agronomically important plant species. Here we report the modification of a fatty acid desaturase 2 gene (FAD2), which encodes an enzyme that catalyzes the desaturation of oleic acid, in Brassica napus cv. Westar using the CRISPR/Cas9 system. Two guide RNAs were designed for BnaA.FAD2.a (FAD2_Aa). Of 22 regenerated shoots with FAD2_Aa editing vectors, three contained mutant alleles. Further analysis revealed that two of three mature plants (Aa1#13 and Aa2#2) contained the mutant alleles. The mutant fad2_Aa allele had a 4-bp deletion, which was inherited by backcross progenies (BC1) in the Aa1#13 line. Furthermore, plants with the fad2_Aa allele without transgenes were selected from the BC1 progenies and plants homozygous for fad2_Aa were then produced by self-crossing these BC1 progenies (BC1S1). Fatty acid composition analysis of their seeds revealed a statistically significant increase in the content of oleic acid compared with that in wild-type seeds. These results showed that the application of the CRISPR/Cas9 system is useful to produce desirable mutant plants with an agronomically suitable phenotype by modifying the metabolic pathway in B. napus.

Ono, R., et al. (2019). "Exosome-mediated horizontal gene transfer occurs in double-strand break repair during genome editing." Commun Biol **2**: 57.

The CRISPR-Cas9 system has been successfully applied in many organisms as a powerful genome-editing tool. Undoubtedly, it will soon be applied to human genome editing, including gene therapy. We have previously reported that unintentional DNA sequences derived from retrotransposons, genomic DNA, mRNA and vectors are captured at double-strand breaks (DSBs) sites when DSBs are introduced by the CRISPR-Cas9 system. Therefore, it is possible that unintentional insertions associated with DSB repair represent a potential risk for human genome editing gene therapies. To address this possibility, comprehensive sequencing of DSB sites was performed. Here, we report that exosome-mediated horizontal gene transfer occurs in DSB repair during genome editing. Exosomes are present in all fluids from living animals, including seawater and breathing mammals, suggesting that exosome-mediated horizontal gene transfer is the driving force behind mammalian genome evolution. The findings of this study highlight an emerging new risk for this leading-edge technology.

Orkin, S. H. (2016). "Recent advances in globin research using genome-wide association studies and gene editing." Ann N Y Acad Sci **1368**(1): 5-10.

A long-sought goal in the hemoglobin field has been an improved understanding of the mechanisms that regulate the switch from fetal (HbF) to adult (HbA) hemoglobin during development. With such knowledge, the hope is that strategies for directed reactivation of HbF in adults could be devised as an approach to therapy for the beta-hemoglobinopathies thalassemia and sickle cell disease. Recent genome-wide association studies (GWAS) led to identification of three loci (BCL11A, HBS1L-MYB, and the beta-globin cluster itself) in which natural genetic variation is correlated with different HbF levels in populations. Here, the central role of BCL11A in control of HbF is reviewed from the perspective of how findings may be translated to gene therapy in the not-too-distant future. This summary traces the evolution of recent studies from the initial recognition of BCL11A through GWAS to identification of critical sequences in an enhancer required for its erythroid-specific expression, thereby highlighting an Achilles heel for genome editing.

Park, C. Y., et al. (2016). "Genome-editing technologies for gene correction of hemophilia." Hum Genet **135**(9): 977-981.

Hemophilia is caused by various mutations in blood coagulation factor genes, including factor VIII (FVIII) and factor IX (FIX), that encode key proteins in the blood clotting pathway. Although the addition of therapeutic genes or infusion of clotting factors may be used to remedy hemophilia's symptoms, no permanent cure for the disease exists. Moreover, patients often develop neutralizing antibodies or experience adverse effects that limit the therapy's benefits. However, targeted gene therapy involving the precise correction of these mutated genes at the genome level using programmable nucleases is a promising strategy. These nucleases can induce double-strand breaks (DSBs) on genomes, and repairs of such induced DSBs by the two cellular repair systems enable a targeted gene correction. Going beyond cultured cell systems, we are now entering the age of direct gene correction in vivo using various delivery tools. Here, we describe the current status of in vivo and ex vivo genome-editing technology related to potential hemophilia gene correction and the prominent issues surrounding its application in patients with monogenic diseases.

Park, C. Y., et al. (2016). "Genome Editing of Structural Variations: Modeling and Gene Correction." Trends Biotechnol **34**(7): 548-561.

The analysis of chromosomal structural variations (SVs), such as inversions and translocations, was made possible by the completion of the human genome project and the development of genome-wide sequencing technologies. SVs contribute to genetic diversity and evolution, although some SVs can cause diseases such as hemophilia A in humans. Genome engineering technology using programmable nucleases (e.g., ZFNs, TALENs, and CRISPR/Cas9) has been rapidly developed, enabling precise and efficient genome editing for SV research. Here, we review advances in modeling and gene correction of SVs, focusing on inversion, translocation, and nucleotide repeat expansion.

Pixley, K. V., et al. (2019). "Genome Editing, Gene Drives, and Synthetic Biology: Will They Contribute to Disease-Resistant Crops, and Who Will Benefit?" Annu Rev Phytopathol **57**: 165-188.

Genetically engineered crops have been grown for more than 20 years, resulting in widespread albeit variable benefits for farmers and consumers. We review current, likely, and potential genetic engineering (GE) applications for the development of disease-resistant crop cultivars. Gene editing, gene drives, and synthetic biology offer novel opportunities to control viral, bacterial, and fungal pathogens, parasitic weeds, and insect vectors of plant pathogens. We conclude that there will be no shortage of GE applications totackle disease resistance and other farmer and consumer priorities for agricultural crops. Beyond reviewing scientific prospects for genetically engineered crops, we address the social institutional forces that are commonly overlooked by biological scientists. Intellectual property regimes, technology regulatory frameworks, the balance of funding between public- and private-sector research, and advocacy by concerned civil society groups interact to define who uses which GE technologies, on which crops, and for the benefit of whom. Ensuring equitable access to the benefits of genetically engineered crops requires affirmative policies, targeted investments, and excellent science.

Poddar, S., et al. (2020). "Efficient isolation of protoplasts from rice calli with pause points and its application in transient gene expression and genome editing assays." Plant Methods **16**(1): 151.

BACKGROUND: An efficient in vivo transient transfection system using protoplasts is an important tool to study gene expression, metabolic pathways, and multiple mutagenesis parameters in plants. Although rice protoplasts can be isolated from germinated seedlings or cell suspension culture, preparation of those donor tissues can be inefficient, time-consuming, and laborious. Additionally, the lengthy process of protoplast isolation and transfection needs to be completed in a single day. RESULTS: Here we report a protocol for the isolation of protoplasts directly from rice calli, without using seedlings or suspension culture. The method is developed to employ discretionary pause points during protoplast isolation and before transfection. Protoplasts maintained within a sucrose cushion partway through isolation, for completion on a subsequent day, per the first pause point, are referred to as S protoplasts. Fully isolated protoplasts maintained in MMG solution for transfection on a subsequent day, per the second pause point, are referred to as M protoplasts. Both S and M protoplasts, 1 day after initiation of protoplast isolation, had minimal loss of viability and transfection efficiency compared to protoplasts 0 days after isolation. S protoplast viability decreases at a lower rate over time than that of M protoplasts and can be used with added flexibility for transient transfection assays and time-course experiments. The protoplasts produced by this method are competent for transfection of both plasmids and ribonucleoproteins (RNPs). Cas9 RNPs were used to demonstrate the utility of these protoplasts to assay genome editing in vivo. CONCLUSION: The current study describes a highly effective and accessible method to isolate protoplasts from callus tissue induced from rice seeds. This

method utilizes donor materials that are resource-efficient and easy to propagate, permits convenience via pause points, and allows for flexible transfection days after protoplast isolation. It provides an advantageous and useful platform for a variety of in vivo transient transfection studies in rice.

Radtke, S., et al. (2020). "Mouse models in hematopoietic stem cell gene therapy and genome editing." Biochem Pharmacol **174**: 113692.

Gene therapy has become an important treatment option for a variety of hematological diseases. The biggest advances have been made with CAR T cells and many of those studies are now FDA approved as a routine treatment for some hematologic malignancies. Hematopoietic stem cell (HSC) gene therapy is not far behind with treatment approvals granted for beta-hemoglobinopathies and adenosine deaminase severe combined immune deficiency (ADA-SCID), and additional approbations currently being sought. With the current pace of research, the significant investment of biotech companies, and the continuously growing toolbox of viral as well as non-viral gene delivery methods, the development of new ex vivo and in vivo gene therapy approaches is at an all-time high. Research in the field of gene therapy has been ongoing for more than 4 decades with big success stories as well as devastating drawbacks along the way. In particular, the damaging effect of uncontrolled viral vector integration observed in the initial gene therapy applications in the 90s led to a more comprehensive upfront safety assessment of treatment strategies. Since the late 90s, an important read-out to comprehensively assess the quality and safety of cell products has come forward with the mouse xenograft model. Here, we review the use of mouse models across the different stages of basic, pre-clinical and translational research towards the clinical application of HSC-mediated gene therapy and editing approaches.

Raikwar, S. P., et al. (2018). "Neuro-Immuno-Gene- and Genome-Editing-Therapy for Alzheimer's Disease: Are We There Yet?" J Alzheimers Dis **65**(2): 321-344.

Alzheimer's disease (AD) is a highly complex neurodegenerative disorder and the current treatment strategies are largely ineffective thereby leading to irreversible and progressive cognitive decline in AD patients. AD continues to defy successful treatment despite significant advancements in the field of molecular medicine. Repeatedly, early promising preclinical and clinical results have catapulted into devastating setbacks leading to multi-billion dollar losses not only to the top pharmaceutical companies but also to the AD patients and their families. Thus, it is very timely to review the progress in the emerging fields of gene therapy and stem cell-based precision medicine. Here, we have made sincere efforts to feature the ongoing progress especially in the field of AD gene therapy and stem cell-based regenerative medicine. Further, we also provide highlights in elucidating the molecular mechanisms underlying AD pathogenesis and describe novel AD therapeutic targets and strategies for the new drug discovery. We hope that the quantum leap in the scientific advancements and improved funding will bolster novel concepts that will propel the momentum toward a trajectory leading to a robust AD patient-specific next generation precision medicine with improved cognitive function and excellent life quality.

Ran, Y., et al. (2018). "Zinc finger nuclease-mediated precision genome editing of an endogenous gene in hexaploid bread wheat (Triticum aestivum) using a DNA repair template." Plant Biotechnol J **16**(12): 2088-2101.

Sequence-specific nucleases have been used to engineer targeted genome modifications in various plants. While targeted gene knockouts resulting in loss of function have been reported with relatively high rates of success, targeted gene editing using an exogenously supplied DNA repair template and site-specific transgene integration has been more challenging. Here, we report the first application of zinc finger nuclease (ZFN)-mediated, nonhomologous end-joining (NHEJ)-directed editing of a native gene in allohexaploid bread wheat to introduce, via a supplied DNA repair template, a specific single amino acid change into the coding sequence of acetohydroxyacid synthase (AHAS) to confer resistance to imidazolinone herbicides. We recovered edited wheat plants having the targeted amino acid modification in one or more AHAS homoalleles via direct selection for resistance to imazamox, an AHAS-inhibiting imidazolinone herbicide. Using a cotransformation strategy based on chemical selection for an exogenous marker, we achieved a 1.2% recovery rate of edited plants having the desired amino acid change and a 2.9% recovery of plants with targeted mutations at the AHAS locus resulting in a loss-of-function gene knockout. The latter results demonstrate a broadly applicable approach to introduce targeted modifications into native genes for nonselectable traits. All ZFN-mediated changes were faithfully transmitted to the next generation.

Richardson, A. O., et al. (2013). "The "fossilized" mitochondrial genome of Liriodendron tulipifera: ancestral gene content and order, ancestral editing sites, and extraordinarily low mutation rate." BMC Biol **11**: 29.

BACKGROUND: The mitochondrial genomes of flowering plants vary greatly in size, gene content, gene order, mutation rate and level of RNA

editing. However, the narrow phylogenetic breadth of available genomic data has limited our ability to reconstruct these traits in the ancestral flowering plant and, therefore, to infer subsequent patterns of evolution across angiosperms. RESULTS: We sequenced the mitochondrial genome of Liriodendron tulipifera, the first from outside the monocots or eudicots. This 553,721 bp mitochondrial genome has evolved remarkably slowly in virtually all respects, with an extraordinarily low genome-wide silent substitution rate, retention of genes frequently lost in other angiosperm lineages, and conservation of ancestral gene clusters. The mitochondrial protein genes in Liriodendron are the most heavily edited of any angiosperm characterized to date. Most of these sites are also edited in various other lineages, which allowed us to polarize losses of editing sites in other parts of the angiosperm phylogeny. Finally, we added comprehensive gene sequence data for two other magnoliids, Magnolia stellata and the more distantly related Calycanthus floridus, to measure rates of sequence evolution in Liriodendron with greater accuracy. The Magnolia genome has evolved at an even lower rate, revealing a roughly 5,000-fold range of synonymous-site divergence among angiosperms whose mitochondrial gene space has been comprehensively sequenced. CONCLUSIONS: Using Liriodendron as a guide, we estimate that the ancestral flowering plant mitochondrial genome contained 41 protein genes, 14 tRNA genes of mitochondrial origin, as many as 7 tRNA genes of chloroplast origin, >700 sites of RNA editing, and some 14 colinear gene clusters. Many of these gene clusters, genes and RNA editing sites have been variously lost in different lineages over the course of the ensuing reverse similar200 million years of angiosperm evolution.

Rocha-Martins, M., et al. (2015). "From Gene Targeting to Genome Editing: Transgenic animals applications and beyond." An Acad Bras Cienc **87**(2 Suppl): 1323-1348.

Genome modification technologies are powerful tools for molecular biology and related areas. Advances in animal transgenesis and genome editing technologies during the past three decades allowed systematic interrogation of gene function that can help model how the genome influences cellular physiology. Genetic engineering via homologous recombination (HR) has been the standard method to modify genomic sequences. Nevertheless, nuclease-guided genome editing methods that were developed recently, such as ZFN, TALEN and CRISPR/Cas, opened new perspectives for biomedical research. Here, we present a brief historical perspective of genome modification methods, focusing on transgenic mice models. Moreover, we describe how new techniques were discovered and improved, present the paradigm shifts and discuss their limitations and applications for biomedical research as well as possible future directions.

Salsman, J., et al. (2017). "CRISPR/Cas9 Gene Editing: From Basic Mechanisms to Improved Strategies for Enhanced Genome Engineering In Vivo." Curr Gene Ther **17**(4): 263-274.

INTRODUCTION: Targeted genome editing using the CRISPR/Cas9 technology is becoming a major area of research due to its high potential for the treatment of genetic diseases. Our understanding of this approach has expanded in recent years yet several new challenges have presented themselves as we explore the boundaries of this exciting new technology. Chief among these is improving the efficiency but also the preciseness of genome editing. The efficacy of CRISPR/Cas9 technology relies in part on the use of one of the major DNA repair pathways, Homologous recombination (HR), which is primarily active in S and G2 phases of the cell cycle. Problematically, the HR potential is highly variable from cell type to cell type and most of the cells of interest to be targeted in vivo for precise genome editing are in a quiescent state. CONCLUSION: In this review, we discuss the recent advancements in improving targeted CRISPR/Cas9 based genome editing and the promising ways of delivering this technology in vivo to the cells of interest.

Sanjurjo-Soriano, C. and V. Kalatzis (2018). "Guiding Lights in Genome Editing for Inherited Retinal Disorders: Implications for Gene and Cell Therapy." Neural Plast **2018**: 5056279.

Inherited retinal dystrophies (IRDs) are a leading cause of visual impairment in the developing world. These conditions present an irreversible dysfunction or loss of neural retinal cells, which significantly impacts quality of life. Due to the anatomical accessibility and immunoprivileged status of the eye, ophthalmological research has been at the forefront of innovative and advanced gene- and cell-based therapies, both of which represent great potential as therapeutic treatments for IRD patients. However, due to a genetic and clinical heterogeneity, certain IRDs are not candidates for these approaches. New advances in the field of genome editing using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated protein (Cas) have provided an accurate and efficient way to edit the human genome and represent an appealing alternative for treating IRDs. We provide a brief update on current gene augmentation therapies for retinal dystrophies. Furthermore, we discuss recent advances in the field of genome editing and stem cell technologies, which

together enable precise and personalized therapies for patients. Lastly, we highlight current technological limitations and barriers that need to be overcome before this technology can become a viable treatment option for patients.

Santosh Kumar, V. V., et al. (2020). "CRISPR-Cas9 mediated genome editing of drought and salt tolerance (OsDST) gene in indica mega rice cultivar MTU1010." Physiol Mol Biol Plants **26**(6): 1099-1110.

Development of abiotic stress tolerant rice cultivars is necessary for sustainable rice production under the scenario of global climate change, dwindling fresh water resources and increase in salt affected areas. Several genes from rice have been functionally validated by using EMS mutants and transgenics. Often, many of these desirable alleles are not available indica rice which is mainly cultivated, and where available, introgression of these alleles into elite cultivars is a time and labour intensive process, in addition to the potential introgression of non-desirable genes due to linkage. CRISPR-Cas technology helps development of elite cultivars with desirable alleles by precision gene editing. Hence, this study was carried out to create mutant alleles of drought and salt tolerance (DST) gene by using CRISPR-Cas9 gene editing in indica rice cv. MTU1010. We used two different gRNAs to target regions of DST protein that might be involved in protein-protein interaction and successfully generated different mutant alleles of DST gene. We selected homozygous dst mutant with 366 bp deletion between the two gRNAs for phenotypic analysis. This 366 bp deletion led to the deletion of amino acid residues from 184 to 305 in frame, and hence the mutant was named as dst (184-305) . The dst (184-305) mutation induced by CRISPR-Cas9 method in DST gene in indica rice cv. MTU1010 phenocopied EMS-induced dst (N69D) mutation reported earlier in japonica cultivar. The dst (184-305) mutant produced leaves with broader width and reduced stomatal density, and thus enhanced leaf water retention under dehydration stress. Our study showed that the reduction in stomatal density in loss of function mutants of dst is, at least, in part due to downregulation of stomatal developmental genes SPCH1, MUTE and ICE1. The Cas9-free dst (184-305) mutant exhibited moderate level tolerance to osmotic stress and high level of salt stress in seedling stage. Thus, dst mutant alleles generated in this study will be useful for improving drought and salt tolerance and grain yield in indica rice cultivars.

Schaeffer, S. M. and P. A. Nakata (2015). "CRISPR/Cas9-mediated genome editing and gene replacement in plants: Transitioning from lab to field." Plant Sci **240**: 130-142.

The CRISPR/Cas9 genome engineering system has ignited and swept through the scientific community like wildfire. Owing largely to its efficiency, specificity, and flexibility, the CRISPR/Cas9 system has quickly become the preferred genome-editing tool of plant scientists. In plants, much of the early CRISPR/Cas9 work has been limited to proof of concept and functional studies in model systems. These studies, along with those in other fields of biology, have led to the development of several utilities of CRISPR/Cas9 beyond single gene editing. Such utilities include multiplexing for inducing multiple cleavage events, controlling gene expression, and site specific transgene insertion. With much of the conceptual CRISPR/Cas9 work nearly complete, plant researchers are beginning to apply this gene editing technology for crop trait improvement. Before rational strategies can be designed to implement this technology to engineer a wide array of crops there is a need to expand the availability of crop-specific vectors, genome resources, and transformation protocols. We anticipate that these challenges will be met along with the continued evolution of the CRISPR/Cas9 system particularly in the areas of manipulation of large genomic regions, transgene-free genetic modification, development of breeding resources, discovery of gene function, and improvements upon CRISPR/Cas9 components. The CRISPR/Cas9 editing system appears poised to transform crop trait improvement.

Schmieder, V., et al. (2018). "Enhanced Genome Editing Tools For Multi-Gene Deletion Knock-Out Approaches Using Paired CRISPR sgRNAs in CHO Cells." Biotechnol J **13**(3): e1700211.

Since the establishment of clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9, powerful strategies for engineering of CHO cell lines have emerged. Nevertheless, there is still room to expand the scope of the CRISPR tool box for further applications to improve CHO cell factories. Here, the authors demonstrate activity of the alternative CRISPR endonuclease Cpf1 in CHO-K1 for the first time and that it can be used in parallel to CRISPR/Cas9 without any interference. Both, Cas9 and Cpf1, can be effectively used for multi-gene engineering with a strategy based on paired single guide RNAs (sgRNAs) for full gene deletions. This strategy also enables the targeting of regulatory regions, which would not respond to the conventional frameshift mutations, as shown by removing the alpha-1,6-Fucosyltransferase 8 (FUT8) promoter resulting in a functional knock-out. FUT8 also served as model to verify that deletion efficiency is size-independent (2-150 kb). To test the suitability for multi-gene approaches in combination with gene deletion, clones harboring triple deletions in beta-1,4-Galactosyltransferase (B4GALT) isozymes

are identified using solely conventional PCR/qPCR. In addition, two bicistronic transcription strategies are implemented to enable unequivocal pairing of sgRNAs: a CHO-derived tRNA linker that works for both, Cas9 and Cpf1, as well as paired sgRNAs in an array format, which can be used with Cpf1 due to its RNA processing ability. These strategies broaden the range of application of CRISPR for novel gene editing approaches in CHO cells and also enable the efficient realization of a genome-wide deletion library.

Sergeeva, D., et al. (2019). "CRISPR/Cas9 as a Genome Editing Tool for Targeted Gene Integration in CHO Cells." Methods Mol Biol **1961**: 213-232.

The emergence of CRISPR/Cas9 system as a precise and affordable method for genome editing has prompted its rapid adoption for the targeted integration of transgenes in Chinese hamster ovary (CHO) cells. Targeted gene integration allows the generation of stable cell lines with a controlled and predictable behavior, which is an important feature for the rational design of cell factories aimed at the large-scale production of recombinant proteins. Here we present the protocol for CRISPR/Cas9-mediated integration of a gene expression cassette into a specific genomic locus in CHO cells using homology-directed DNA repair.

Serif, M., et al. (2018). "One-step generation of multiple gene knock-outs in the diatom Phaeodactylum tricornutum by DNA-free genome editing." Nat Commun **9**(1): 3924.

Recently developed transgenic techniques to explore and exploit the metabolic potential of microalgae present several drawbacks associated with the delivery of exogenous DNA into the cells and its subsequent integration at random sites within the genome. Here, we report a highly efficient multiplex genome-editing method in the diatom Phaeodactylum tricornutum, relying on the biolistic delivery of CRISPR-Cas9 ribonucleoproteins coupled with the identification of two endogenous counter-selectable markers, PtUMPS and PtAPT. First, we demonstrate the functionality of RNP delivery by positively selecting the disruption of each of these genes. Then, we illustrate the potential of the approach for multiplexing by generating double-gene knock-out strains, with 65% to 100% efficiency, using RNPs targeting one of these markers and PtAureo1a, a photoreceptor-encoding gene. Finally, we created triple knock-out strains in one step by delivering six RNP complexes into Phaeodactylum cells. This approach could readily be applied to other hard-to-transfect organisms of biotechnological interest.

Sevestre, F., et al. (2020). "Facilitating gene editing in potato: a Single-Nucleotide Polymorphism (SNP) map of the Solanum tuberosum L. cv. Desiree genome." Sci Rep **10**(1): 2045.

Genome editing is a powerful tool for plant functional genomics allowing for multiallelic targeted mutagenesis. The recent development of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR associated protein 9 (Cas9) systems for gene editing in plants allows for simple, cost-effective introduction of site-specific double-stranded DNA breaks. The nuclear genomes of a homozygous doubled-monoploid potato clone (DM) and a heterozygous diploid clone (RH) have been sequenced in 2011. However, common potato cultivars display a highly heterozygous autotetraploid genome thus complicating target design for tetra-allelic gene editing. Here, we report on the SNP physical map of the widely used Solanum tuberosum L. cv. Desiree and on the position of the diverse indels providing an essential tool for target design in genome editing approaches. We used this tool for designing a specific gRNA and successfully knocking-out a newly discovered starch synthase gene (SS6) in potato. Resequencing data are publicly available at the Sequence Read Archive of the NCBI (accession number: PRJNA507597) and will represent a valuable resource for functional genomic studies of various metabolic pathways, cell and plant physiology as well as high-throughput reverse genetics in potato.

Shtratnikova, V. Y., et al. (2020). "Mitochondrial genome of the nonphotosynthetic mycoheterotrophic plant Hypopitys monotropa, its structure, gene expression and RNA editing." PeerJ **8**: e9309.

Heterotrophic plants-plants that have lost the ability to photosynthesize-are characterized by a number of changes at all levels of organization. Heterotrophic plants are divided into two large categories-parasitic and mycoheterotrophic (MHT). The question of to what extent such changes are similar in these two categories is still open. The plastid genomes of nonphotosynthetic plants are well characterized, and they exhibit similar patterns of reduction in the two groups. In contrast, little is known about the mitochondrial genomes of MHT plants. We report the structure of the mitochondrial genome of Hypopitys monotropa, a MHT member of Ericaceae, and the expression of its genes. In contrast to its highly reduced plastid genome, the mitochondrial genome of H. monotropa is larger than that of its photosynthetic relative Vaccinium macrocarpon, and its complete size is ~810 Kb. We observed an unusually long repeat-rich structure of the genome that suggests the existence of linear fragments. Despite this unique feature, the gene content of the H. monotropa mitogenome is typical of

flowering plants. No acceleration of substitution rates is observed in mitochondrial genes, in contrast to previous observations in parasitic non-photosynthetic plants. Transcriptome sequencing revealed the trans-splicing of several genes and RNA editing in 33 of 38 genes. Notably, we did not find any traces of horizontal gene transfer from fungi, in contrast to plant parasites, which extensively integrate genetic material from their hosts.

Shuvalov, O., et al. (2015). "Current genome editing tools in gene therapy: new approaches to treat cancer." Curr Gene Ther **15**(5): 511-529.

Gene therapy suggests a promising approach to treat genetic diseases by applying genes as pharmaceuticals. Cancer is a complex disease, which strongly depends on a particular genetic make-up and hence can be treated with gene therapy. From about 2,000 clinical trials carried out so far, more than 60% were cancer targeted. Development of precise and effective gene therapy approaches is intimately connected with achievements in the molecular biology techniques. The field of gene therapy was recently revolutionized by the introduction of "programmable" nucleases, including ZFNs, TALENs, and CRISPR, which target specific genomic loci with high efficacy and precision. Furthermore, when combined with DNA transposons for the delivery purposes into cells, these programmable nucleases represent a promising alternative to the conventional viral-mediated gene delivery. In addition to "programmable" nucleases, a new class of TALE- and CRISPR-based "artificial transcription effectors" has been developed to mediate precise regulation of specific genes. In sum, these new molecular tools may be used in a wide plethora of gene therapy strategies. This review highlights the current status of novel genome editing tools and discusses their suitability and perspectives in respect to cancer gene therapy studies.

Si, X., et al. (2020). "Manipulating gene translation in plants by CRISPR-Cas9-mediated genome editing of upstream open reading frames." Nat Protoc **15**(2): 338-363.

Gene expression is regulated by multiple processes, and the translation of mRNAs into proteins is an especially critical step. Upstream open reading frames (uORFs) are widespread cis-elements in eukaryotic genes that usually suppress the translation of downstream primary ORFs (pORFs). Here, we describe a protocol for fine-tuning gene translation in plants by editing endogenous uORFs with the CRISPR-Cas9 system. The method we present readily yields transgene-free uorf mutant offspring. We provide detailed protocols for predicting uORFs and testing their effects on downstream pORFs using a dual-luciferase reporter system, designing and constructing single guide RNA (sgRNA)-Cas9 vectors, identifying transgene-free uorf mutants, and finally comparing the mRNA, protein and phenotypic levels of target genes in uorf mutants and controls. Predicting uORFs and confirming their effects in protoplasts takes only 2-3 weeks, and transgene-free mutants with edited target uORFs controlling different levels of pORF translation can be obtained within 4 months. Unlike previous methods, our strategy achieves fine-tuning of gene translation in transgene-free derivatives, which accelerates the analysis of gene function and the improvement of crop traits.

Skakic, A., et al. (2019). "CRISPR/Cas9 genome editing of SLC37A4 gene elucidates the role of molecular markers of endoplasmic reticulum stress and apoptosis in renal involvement in glycogen storage disease type Ib." Gene **703**: 17-25.

Glycogen storage disease type Ib (GSD Ib) is an autosomal recessive disorder, caused by a deficiency of ubiquitously expressed SLC37A4 protein. Deficiency of SLC37A4 leads to abnormal storage of glycogen in the liver and kidneys, resulting in long-term complications of renal disease and hepatocellular adenomas, whose mechanisms are poorly understood. Molecular markers of the adaptive responses to the metabolic stress caused by a deficiency of SLC37A4, such as markers related to the endoplasmic reticulum (ER) stress and unfolded protein response (UPR), have not been extensively studied. The aim of this study was to investigate the expression of molecular markers of the UPR response and apoptosis related to a deficiency of SLC37A4 in kidney cells. For that purpose, we intended to establish a human kidney cell model system for GSD Ib. The novel variant c.248G>A, found in GSD Ib patients, was introduced into the Flp-InT-REx-293 cell line using CRISPR/Cas9-mediated precise gene editing method, resulting in significant decrease of SLC37A4 gene expression. In this model system we used RT-qPCR analysis to investigate the expression of molecular markers of the UPR response (ATF4, DDIT3, HSPA5, and XBP1s) and apoptosis (BCL2, BAX). We demonstrated that under chronic metabolic stress conditions caused by SLC37A4 deficiency, the ER stress-induced UPR was triggered, resulting in suppression of the UPR molecular markers and cell survival promotion (decreased expression levels of ATF4, DDIT3, HSPA5, with the exception of XBP1s). However, persistent metabolic stress overrides an adaptation and induces apoptosis through increased expression of pro-apoptotic markers (decreased ratio of BCL2/BAX genes). We established a cellular model system characterized by a deficiency of SLC37A4, which presents pathological manifestations of GSD Ib in the kidney. Expression analysis in a novel model

system supports the hypothesis that renal dysfunction in the GSD Ib is partly due to the ER stress and increased apoptosis.

Suenami, S., et al. (2018). "Kenyon Cell Subtypes/Populations in the Honeybee Mushroom Bodies: Possible Function Based on Their Gene Expression Profiles, Differentiation, Possible Evolution, and Application of Genome Editing." Front Psychol **9**: 1717.

Mushroom bodies (MBs), a higher-order center in the honeybee brain, comprise some subtypes/populations of interneurons termed as Kenyon cells (KCs), which are distinguished by their cell body size and location in the MBs, as well as their gene expression profiles. Although the role of MBs in learning ability has been studied extensively in the honeybee, the roles of each KC subtype and their evolution in hymenopteran insects remain mostly unknown. This mini-review describes recent progress in the analysis of gene/protein expression profiles and possible functions of KC subtypes/populations in the honeybee. Especially, the discovery of novel KC subtypes/populations, the "middle-type KCs" and "KC population expressing FoxP," necessitated a redefinition of the KC subtype/population. Analysis of the effects of inhibiting gene function in a KC subtype-preferential manner revealed the function of the gene product as well as of the KC subtype where it is expressed. Genes expressed in a KC subtype/population-preferential manner can be used to trace the differentiation of KC subtypes during the honeybee ontogeny and the possible evolution of KC subtypes in hymenopteran insects. Current findings suggest that the three KC subtypes are unique characteristics to the aculeate hymenopteran insects. Finally, prospects regarding future application of genome editing for the study of KC subtype functions in the honeybee are described. Genes expressed in a KC subtype-preferential manner can be good candidate target genes for genome editing, because they are likely related to highly advanced brain functions and some of them are dispensable for normal development and sexual maturation in honeybees.

Surun, D., et al. (2018). "High Efficiency Gene Correction in Hematopoietic Cells by Donor-Template-Free CRISPR/Cas9 Genome Editing." Mol Ther Nucleic Acids **10**: 1-8.

The CRISPR/Cas9 prokaryotic adaptive immune system and its swift repurposing for genome editing enables modification of any prespecified genomic sequence with unprecedented accuracy and efficiency, including targeted gene repair. We used the CRISPR/Cas9 system for targeted repair of patient-specific point mutations in the Cytochrome b-245 heavy chain gene (CYBB), whose inactivation causes chronic granulomatous disease (XCGD)-a life-threatening immunodeficiency disorder characterized by the inability of neutrophils and macrophages to produce microbicidal reactive oxygen species (ROS). We show that frameshift mutations can be effectively repaired in hematopoietic cells by non-integrating lentiviral vectors carrying RNA-guided Cas9 endonucleases (RGNs). Because about 25% of most inherited blood disorders are caused by frameshift mutations, our results suggest that up to a quarter of all patients suffering from monogenic blood disorders could benefit from gene therapy employing personalized, donor template-free RGNs.

Suzuki, K. and J. C. Izpisua Belmonte (2018). "In vivo genome editing via the HITI method as a tool for gene therapy." J Hum Genet **63**(2): 157-164.

Using genome-editing technologies to correct specific mutations represents a potentially transformative new approach for treating genetic disorders. Despite rapid advances in the field of genome editing, it is still unclear whether the long-standing goal of in vivo targeted transgene integration is feasible. This is primarily because current tools are inefficient. In particular, current technologies are incapable of targeted gene knock-in in non-dividing cells, the major building blocks of adult tissues. This poses a significant barrier for developing therapeutic strategies to treat a broad range of devastating genetic disorders. Recently, our group has developed a unique CRISPR/Cas9-based strategy, termed homology-independent targeted insertion (HITI), which enables targeted gene insertion in non-dividing cells, both in vitro and in vivo. This review will summarize current progress in developing this technology, and discuss the potential impact of HITI-based gene-correction therapies.

Suzuki, K., et al. (2019). "Precise in vivo genome editing via single homology arm donor mediated intron-targeting gene integration for genetic disease correction." Cell Res **29**(10): 804-819.

In vivo genome editing represents a powerful strategy for both understanding basic biology and treating inherited diseases. However, it remains a challenge to develop universal and efficient in vivo genome-editing tools for tissues that comprise diverse cell types in either a dividing or non-dividing state. Here, we describe a versatile in vivo gene knock-in methodology that enables the targeting of a broad range of mutations and cell types through the insertion of a minigene at an intron of the target gene locus using an intracellularly linearized single homology arm donor. As a proof-of-concept, we focused on a mouse model of premature-aging caused by a dominant point

mutation, which is difficult to repair using existing in vivo genome-editing tools. Systemic treatment using our new method ameliorated aging-associated phenotypes and extended animal lifespan, thus highlighting the potential of this methodology for a broad range of in vivo genome-editing applications.

Suzuki, T., et al. (2020). "Preparation of the standard cell lines for reference mutations in cancer gene-panels by genome editing in HEK 293 T/17 cells." Genes Environ **42**: 8.

Background: Next Generation Sequencer (NGS) is a powerful tool for a high-throughput sequencing of human genome. It is important to ensure reliability and sensitivity of the sequence data for a clinical use of the NGS. Various cancer-related gene panels such as Oncomine or NCC OncoPanel have been developed and used for clinical studies. Because these panels contain multiple genes, it is difficult to ensure the performance of mutation detection for every gene. In addition, various platforms of NGS are developed and their cross-platform validation has become necessity. In order to create mutant standards in a defined background, we have used CRISPR/Cas9 genome-editing system in HEK 293 T/17 cells. Results: Cancer-related genes that are frequently used in NGS-based cancer panels were selected as the target genes. Target mutations were selected based on their frequency reported in database, and clinical significance and on the applicability of CRISPR/Cas9 by considering distance from PAM site, and off-targets. We have successfully generated 88 hetero- and homozygous mutant cell lines at the targeted sites of 36 genes representing a total of 125 mutations. Conclusions: These knock-in HEK293T/17 cells can be used as the reference mutant standards with a steady and continuous supply for NGS-based cancer panel tests from the JCRB cell bank. In addition, these cell lines can provide a tool for the functional analysis of targeted mutations in cancer-related genes in the isogenic background.

Tabebordbar, M., et al. (2017). Therapeutic Gene Editing in Muscles and Muscle Stem Cells. Genome Editing in Neurosciences. R. Jaenisch, F. Zhang and F. Gage. Cham (CH)**:** 103-123.

Duchenne muscular dystrophy (DMD) is a devastating, degenerative muscle disease that affects ~1 in every 3500 male births. DMD arises from mutations in the DMD gene that prevent expression of its encoded protein, Dystrophin (Burghes et al. Nature 328:434-437, 1987). Interestingly, patients with Dmd mutations that delete certain segments of the Dystrophin coding region, but maintain protein reading frame, have a much milder form of the disease, known as Becker Muscular Dystrophy (BMD). This observation has spurred interest in developing "exon skipping" strategies in which certain mutation-containing or mutation-adjacent Dmd exons are intentionally removed in order to restore protein reading frame, and thereby Dystrophin expression, in DMD patients (Beroud et al. Hum Mutat 28:196-202, 2007; Yokota et al. Expert Opin Biol Ther 7:831-842, 2007).

Takadera, M., et al. (2020). "Phenotypic characterization with somatic genome editing and gene transfer reveals the diverse oncogenicity of ependymoma fusion genes." Acta Neuropathol Commun **8**(1): 203.

Recurrent RELA and YAP1 fusions are intimately associated with tumorigenesis in supratentorial ependymomas. Chromothripsis and focal copy number alterations involving 11q are hallmarks of these tumors. However, it is unknown whether the chromosomal alterations are a direct causal event resulting in fusion transcripts. In addition, the biological significance of the RELA fusion variants and YAP1 fusions is not yet fully characterized. In this study, we generated gene rearrangements on 11q with the CRISPR/Cas9 system and investigated the formation of oncogenic ependymoma fusion genes. Further, we examined the oncogenic potential of RELA fusion variants and YAP1 fusions in a lentiviral gene transfer model. We observed that endogenous RELA fusion events were successfully induced by CRISPR/Cas9-mediated genome rearrangement in cultured cells. In vivo genome editing in mouse brain resulted in the development of ependymoma-like brain tumors that harbored the Rela fusion gene. All RELA fusion variants tested, except a variant lacking the Rel homology domain, were able to induce tumor formation, albeit with different efficacy. Furthermore, expression of YAP1-FAM118B and YAP1-MAMLD1 fusions induced the formation of spindle-cell-like tumors at varying efficacy. Our results indicate that chromosomal rearrangements involving the Rela locus are the causal event for the formation of Rela fusion-driven ependymomas in mice. Furthermore, the type of RELA. fusion might affect the aggressiveness of tumors and that the Rel homology domain is essential for the oncogenic functions of RELA. fusions. The YAP1 fusion genes are also oncogenic when expressed in mice.

Tan, W., et al. (2016). "Gene targeting, genome editing: from Dolly to editors." Transgenic Res **25**(3): 273-287.

One of the most powerful strategies to investigate biology we have as scientists, is the ability to transfer genetic material in a controlled and deliberate manner between organisms. When applied to

livestock, applications worthy of commercial venture can be devised. Although initial methods used to generate transgenic livestock resulted in random transgene insertion, the development of SCNT technology enabled homologous recombination gene targeting strategies to be used in livestock. Much has been accomplished using this approach. However, now we have the ability to change a specific base in the genome without leaving any other DNA mark, with no need for a transgene. With the advent of the genome editors this is now possible and like other significant technological leaps, the result is an even greater diversity of possible applications. Indeed, in merely 5 years, these 'molecular scissors' have enabled the production of more than 300 differently edited pigs, cattle, sheep and goats. The advent of genome editors has brought genetic engineering of livestock to a position where industry, the public and politicians are all eager to see real use of genetically engineered livestock to address societal needs. Since the first transgenic livestock reported just over three decades ago the field of livestock biotechnology has come a long way-but the most exciting period is just starting.

Tang, J. X., et al. (2018). "CRISPR/Cas9-mediated genome editing induces gene knockdown by altering the pre-mRNA splicing in mice." BMC Biotechnol **18**(1): 61.

BACKGROUND: Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated protein 9 (CRISPR/Cas9) has been wildly used to generate gene knockout models through inducing indels causing frame-shift. However, there are few studies concerning the post-transcript effects caused by CRISPR-mediated genome editing. RESULTS: In the present study, we showed that gene knockdown model also could be generated using CRISPR-mediated gene editing by disrupting the boundary of exon and intron in mice (C57BL/6 J). CRISPR induced indel at the boundary of exon and intron (5' splice site) caused alternative splicing and produced multiple different mRNAs, most of these mRNAs introduced premature termination codon causing down expression of the gene. CONCLUSIONS: These results showed that alternative splicing mutants were able to generate through CRISPR-mediated genome editing by deleting the boundary of exon and intron causing disruption of 5' splice site. Although alternative splicing was an unexpected outcome, this finding could be developed as a technology to generate gene knockdown models or to investigate pre-mRNA splicing.

Toda, E. and T. Okamoto (2020). "Gene Expression and Genome Editing Systems by Direct Delivery of Macromolecules Into Rice Egg Cells and Zygotes." Bio Protoc **10**(14): e3681.

Polyethylene glycol calcium (PEG-Ca(2+))-mediated transfection allows rapid and efficient examination to analyze diverse cellular functions of genes of interest. In plant cells, macromolecules, such as DNA, RNA and protein, are delivered into protoplasts derived from somatic tissues or calli via PEG-Ca(2+) transfection. To broaden and develop the scope of investigations using plant gametes and zygotes, a procedure for direct delivery of macromolecules into these cells has recently been established using PEG-Ca(2+) transfection. This PEG-Ca(2+)-mediated delivery into rice egg cells/zygotes consists of four microtechniques, (i) isolation of gametes, (ii) production of zygotes by electrofusion of gametes, (iii) PEG-Ca(2+)-mediated delivery of macromolecules into isolated egg cells or produced zygotes, and (iv) culture and subsequent analyses of the transfected egg cells/zygotes. Because the full protocol for microtechniques (i) and (ii) have already been reported in Toda et al., 2016 , microtechniques (iii) and (iv) are mainly described in this protocol.

Todokoro, T., et al. (2020). "Identification of a novel pyrithiamine resistance marker gene thiI for genome co-editing in Aspergillus oryzae." J Biosci Bioeng **130**(3): 227-232.

Marker genes are essential for gene modification and genome editing of microorganisms. In Aspergillus oryzae, a widely used host for enzyme production, only a few marker genes can be used for positive selection. One of these genes, the pyrithiamine (PT) resistance marker gene thiA, is not useful for CRISPR/Cas9 genome editing because of its unique resistance-conferring mechanism. In this study, a novel PT resistance marker was investigated considering its potential applications in genome editing. A mutant resistant to PT was selected from UV-mutagenized A. oryzae RIB40. Whole genome analysis was conducted on the mutants, and a novel candidate gene for PT resistance was identified. This candidate gene exhibited similarity to the thiamine transporter gene thi9 of Schizosaccharomyces pombe and was designated as thiI. A thiI loss-of-function mutant was generated using the CRISPR/Cas9 genome editing system to investigate its effect on PT resistance. This mutant showed PT resistance and exhibited no growth defect or auxotrophy. The thiI gene was further investigated for its use as a selection marker in genome co-editing. Ribonucleoprotein complex comprising recombinant Cas9 nuclease and sgRNA targeting thiI or another target gene (wA or sreA) was prepared and simultaneously introduced into A. oryzae RIB40. thiI and target gene double loss-of-function mutants were efficiently selected on PT-containing medium. thiI was

shown to be a useful marker gene in A. oryzae for use in genome editing. This study is expected to provide insights, which will promote basic research and industrial applications of A. oryzae.

Ulman, A., et al. (2020). "Genome Editing of the SNAI1 Gene in Rhabdomyosarcoma: A Novel Model for Studies of Its Role." Cells **9**(5).

Genome editing (GE) tools and RNA interference technology enable the modulation of gene expression in cancer research. While GE mediated by clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 or transcription activator-like effector nucleases (TALEN) activity can be used to induce gene knockouts, shRNA interacts with the targeted transcript, resulting in gene knockdown. Here, we compare three different methods for SNAI1 knockout or knockdown in rhabdomyosarcoma (RMS) cells. RMS is the most common sarcoma in children and its development has been previously associated with SNAI1 transcription factor activity. To investigate the role of SNAI1 in RMS development, we compared CRISPR/Cas9, TALEN, and shRNA tools to identify the most efficient tool for the modulation of SNAI1 expression with biological effects. Subsequently, the genome sequence, transcript levels, and protein expression of SNAI1 were evaluated. The modulation of SNAI1 using three different approaches affected the morphology of the cells and modulated the expression of myogenic factors and HDAC1. Our study revealed a similar effectiveness of the tested methods. Nevertheless, the low efficiency of the GE tools was a limiting factor in obtaining biallelic gene knockouts. To conclude, we established and characterized three different models of SNAI1 knockout and knockdown that might be used in further studies investigating the role of SNAI1 in RMS.

Upadhyay, S. K., et al. (2013). "RNA-guided genome editing for target gene mutations in wheat." G3 (Bethesda) **3**(12): 2233-2238.

The clustered, regularly interspaced, short palindromic repeats (CRISPR) and CRISPR-associated protein (Cas) system has been used as an efficient tool for genome editing. We report the application of CRISPR-Cas-mediated genome editing to wheat (Triticum aestivum), the most important food crop plant with a very large and complex genome. The mutations were targeted in the inositol oxygenase (inox) and phytoene desaturase (pds) genes using cell suspension culture of wheat and in the pds gene in leaves of Nicotiana benthamiana. The expression of chimeric guide RNAs (cgRNA) targeting single and multiple sites resulted in indel mutations in all the tested samples. The expression of Cas9 or sgRNA alone did not cause any mutation. The expression of duplex cgRNA with Cas9 targeting two sites in the same gene resulted in deletion of DNA fragment between the targeted sequences. Multiplexing the cgRNA could target two genes at one time. Target specificity analysis of cgRNA showed that mismatches at the 3' end of the target site abolished the cleavage activity completely. The mismatches at the 5' end reduced cleavage, suggesting that the off target effects can be abolished in vivo by selecting target sites with unique sequences at 3' end. This approach provides a powerful method for genome engineering in plants.

van Leeuwe, T. M., et al. (2019). "Efficient marker free CRISPR/Cas9 genome editing for functional analysis of gene families in filamentous fungi." Fungal Biol Biotechnol **6**: 13.

Background: CRISPR/Cas9 mediated genome editing has expedited the way of constructing multiple gene alterations in filamentous fungi, whereas traditional methods are time-consuming and can be of mutagenic nature. These developments allow the study of large gene families that contain putatively redundant genes, such as the seven-membered family of crh-genes encoding putative glucan-chitin crosslinking enzymes involved in cell wall biosynthesis. Results: Here, we present a CRISPR/Cas9 system for Aspergillus niger using a non-integrative plasmid, containing a selection marker, a Cas9 and a sgRNA expression cassette. Combined with selection marker free knockout repair DNA fragments, a set of the seven single knockout strains was obtained through homology directed repair (HDR) with an average efficiency of 90%. Cas9-sgRNA plasmids could effectively be cured by removing selection pressure, allowing the use of the same selection marker in successive transformations. Moreover, we show that either two or even three separate Cas9-sgRNA plasmids combined with marker-free knockout repair DNA fragments can be used in a single transformation to obtain double or triple knockouts with 89% and 38% efficiency, respectively. By employing this technique, a seven-membered crh-gene family knockout strain was acquired in a few rounds of transformation; three times faster than integrative selection marker (pyrG) recycling transformations. An additional advantage of the use of marker-free gene editing is that negative effects of selection marker gene expression are evaded, as we observed in the case of disrupting virtually silent crh family members. Conclusions: Our findings advocate the use of CRISPR/Cas9 to create multiple gene deletions in both a fast and reliable way, while simultaneously omitting possible locus-dependent-side-effects of poor auxotrophic marker expression.

Wang, D. C. and X. Wang (2019). "Off-target genome editing: A new discipline of gene science and a new class of medicine." Cell Biol Toxicol **35**(3): 179-183.

With an increasing growth of genome editing, off-target effects such as non-specific genetic modifications resulting from the designed process of genome editing become a new discipline of gene science and new class medicine. The degree of short-term and long-term side effects and toxicity or dynamics of the primary and secondary off-target genome editing varies with the application of different methodologies of gene editing and measuring, readouts of genetic modifications, or comparison reference. Measurements of dynamic off-target effects caused directly or indirectly by genome editing are critical in clinical application of gene editing. The quality of genome editing methods is one of the decisive factors in the occurrence of off-target effects. Mechanisms by which off-target effects of genome editing occurs are more complex and comprehensive than we expected. The heterogeneity of off-target effects of gene-edited cells at single-cell levels should be defined during the development and formation of cell clusters. In addition to off-target effects on gene-edited cells per se, alterations of gene sequence, structure, dimension, and function of related regulators caused by off-target effects may also influence intercellular communications and interactions between gene-edited cells, between gene-edited cells and non-edited cells, or between non-edited cells. Thus, controlling, measuring, defining, categorizing, and predicting off-target genome editing need to be standardized and prioritized before clinical application of gene editing.

Wang, J., et al. (2015). "TALENs-mediated gene disruption of FLT3 in leukemia cells: Using genome-editing approach for exploring the molecular basis of gene abnormality." Sci Rep **5**: 18454.

Novel analytic tools are needed to elucidate the molecular basis of leukemia-relevant gene mutations in the post-genome era. We generated isogenic leukemia cell clones in which the FLT3 gene was disrupted in a single allele using TALENs. Isogenic clones with mono-allelic disrupted FLT3 were compared to an isogenic wild-type control clone and parental leukemia cells for transcriptional expression, downstream FLT3 signaling and proliferation capacity. The global gene expression profiles of mutant K562 clones and corresponding wild-type controls were compared using RNA-seq. The transcriptional levels and the ligand-dependent autophosphorylation of FLT3 were decreased in the mutant clones. TALENs-mediated FLT3 haplo-insufficiency impaired cell proliferation and colony formation in vitro. These inhibitory effects were maintained in vivo, improving the survival of NOD/SCID mice transplanted with mutant K562 clones. Cluster analysis revealed that the gene expression pattern of isogenic clones was determined by the FLT3 mutant status rather than the deviation among individual isogenic clones. Differentially expressed genes between the mutant and wild-type clones revealed an activation of nonsense-mediated decay pathway in mutant K562 clones as well as an inhibited FLT3 signaling. Our data support that this genome-editing approach is a robust and generally applicable platform to explore the molecular bases of gene mutations.

Wang, J., et al. (2017). "CRISPR/Cas9 mediated genome editing of Helicoverpa armigera with mutations of an ABC transporter gene HaABCA2 confers resistance to Bacillus thuringiensis Cry2A toxins." Insect Biochem Mol Biol **87**: 147-153.

High levels of resistance to Bt toxin Cry2Ab have been identified to be genetically linked with loss of function mutations of an ABC transporter gene (ABCA2) in two lepidopteran insects, Helicoverpa armigera and Helicoverpa punctigera. To further confirm the causal relationship between the ABCA2 gene (HaABCA2) and Cry2Ab resistance in H. armigera, two HaABCA2 knockout strains were created from the susceptible SCD strain with the CRISPR/Cas9 genome editing system. One strain (SCD-A2KO1) is homozygous for a 2-bp deletion in exon 2 of HaABCA2 created by non-homologous end joining (NHEJ). The other strain (SCD-A2KO2) is homozygous for a 5-bp deletion in exon 18 of HaABCA2 made by homology-directed repair (HDR), which was produced to mimic the r2 resistance allele of a field-derived Cry2Ab-resistant strain from Australia. Both knockout strains obtained high levels of resistance to both Cry2Aa (>120-fold) and Cry2Ab (>100-fold) compared with the original SCD strain, but no or very limited resistance to Cry1Ac (<4-fold). Resistance to Cry2Ab in both knockouts is recessive, and genetic complementary tests confirmed Cry2Ab resistance alleles are at the same locus (i.e. HaABCA2) for the two strains. Brush border membrane vesicles (BBMVs) of midguts from both knockout strains lost binding with Cry2Ab, but maintained the same binding with Cry1Ac as the SCD strain. In vivo functional evidence from this study demonstrates knockout of HaABCA2 confers high levels of resistance to both Cry2Aa and Cry2Ab, confirming that HaABCA2 plays a key role in mediating toxicity of both Cry2Aa and Cry2Ab against H. armigera.

Wang, J., et al. (2020). "Efficient CRISPR/Cas9-Mediated Gene Editing in an Interspecific Hybrid Poplar With a Highly Heterozygous Genome." Front Plant Sci **11**: 996.

Although the CRISPR/Cas9 system has been widely used for crop breeding, its application for the genetic improvement of trees has been limited, partly because of the outcrossing nature and substantial genomic heterozygosity of trees. Shanxin yang (Populus davidiana x P. bolleana), is a commercially important poplar clone that is widely grown in northern China. An established transformation protocol for this interspecific hybrid enables researchers to simultaneously investigate the efficiency and specificity of the CRISPR/Cas9-mediated manipulation of a highly heterozygous genome. Using the phytoene desaturase gene (PDS) as an example, we revealed that the CRISPR/Cas9 system could efficiently edit the Shanxin yang genome. Two sgRNAs were designed and incorporated into a single binary vector containing the Cas9 expression cassette. Among 62 independent transgenic lines, 85.5% exhibited an exclusively albino phenotype, revealing the total loss of PDS function. The Illumina sequencing results confirmed the targeted mutation of PdbPDS homologs induced by CRISPR/Cas9, and small insertions/deletions were the most common mutations. Biallelic and homozygous knockout mutations were detected at both target sites of the T0 transformants. Off-target activity was detected for sgRNA2 with a frequency of 3.2%. Additionally, the SNP interference of targeting specificity was assessed based on the sequence variation among PdbPDS homologs. A single mismatch at 19- or 10-bp from the PAM was tolerated by the CRISPR/Cas9 system. Therefore, multiple homologous genes were simultaneously edited despite the presence of a mismatch between the sgRNA and the target site. The establishment of a viable CRISPR/Cas9-based strategy for editing the Shanxin yang genome will not only accelerate the breeding process, but may also be relevant for other economically or scientifically important non-model plants species.

Wang, L., et al. (2015). "Large genomic fragment deletion and functional gene cassette knock-in via Cas9 protein mediated genome editing in one-cell rodent embryos." Sci Rep **5**: 17517.

The CRISPR-Cas RNA-guided system has versatile uses in many organisms and allows modification of multiple target sites simultaneously. Generating novel genetically modified mouse and rat models is one valuable application of this system. Through the injection of Cas9 protein instead of mRNA into embryos, we observed fewer off-target effects of Cas9 and increased point mutation knock-in efficiency. Large genomic DNA fragment (up to 95 kb) deletion mice were generated for in vivo study of lncRNAs and gene clusters. Site-specific insertion of a 2.7 kb CreERT2 cassette into the mouse Nfatc1 locus allowed labeling and tracing of hair follicle stem cells. In addition, we combined the Cre-Loxp system with a gene-trap strategy to insert a GFP reporter in the reverse orientation into the rat Lgr5 locus, which was later inverted by Cre-mediated recombination, yielding a conditional knockout/reporter strategy suitable for mosaic mutation analysis.

Wang, M., et al. (2017). "Non-viral delivery of genome-editing nucleases for gene therapy." Gene Ther **24**(3): 144-150.

Manipulating the genetic makeup of mammalian cells using programmable nuclease-based genome-editing technology has recently evolved into a powerful avenue that holds great potential for treating genetic disorders. There are four types of genome-editing nucleases, including meganucleases, zinc finger nucleases, transcription activator-like effector nucleases and clustered, regularly interspaced, short palindromic repeat-associated nucleases such as Cas9. These nucleases have been harnessed to introduce precise and specific changes of the genome sequence at virtually any genome locus of interest. The therapeutic relevance of these genome-editing technologies, however, is challenged by the safe and efficient delivery of nuclease into targeted cells. Herein, we summarize recent advances that have been made on non-viral delivery of genome-editing nucleases. In particular, we focus on non-viral delivery of Cas9/sgRNA ribonucleoproteins for genome editing. In addition, the future direction for developing non-viral delivery of programmable nucleases for genome editing is discussed.

Wang, M., et al. (2018). "From Genetic Stock to Genome Editing: Gene Exploitation in Wheat." Trends Biotechnol **36**(2): 160-172.

Bread wheat (Triticum aestivum) ranks as one of our most important staple crops. However, its hexaploid nature has complicated our understanding of the genetic bases underlying many of its traits. Historically, functional genetic studies in wheat have focused on identifying natural variations and have contributed to assembling and enriching its genetic stock. Recently, mold-breaking advances in whole genome sequencing, exome-capture based mutant libraries, and genome editing have revolutionized strategies for genetic research in wheat. We review new trends in wheat functional genetic studies along with germplasm conservation and innovation, including the relevance of genetic stocks, and the application of sequencing-based mutagenesis and genome editing. We also highlight the potential of multiplex genome editing toolkits in addressing species-specific challenges in wheat.

Wang, S., et al. (2018). "Complete chloroplast genome sequence of Betula platyphylla: gene organization, RNA editing, and comparative and phylogenetic analyses." BMC Genomics **19**(1): 950.

BACKGROUND: Betula platyphylla is a common tree species in northern China that has high economic and medicinal value. Our laboratory has been devoted to genome research on B. platyphylla for approximately 10 years. As primary organelle genomes, the complete genome sequences of chloroplasts are important to study the divergence of species, RNA editing and phylogeny. In this study, we sequenced and analyzed the complete chloroplast (cp) genome sequence of B. platyphylla. RESULTS: The complete cp genome of B. platyphylla was 160,518 bp in length, which included a pair of inverted repeats (IRs) of 26,056 bp that separated a large single copy (LSC) region of 89,397 bp and a small single copy (SSC) region of 19,009 bp. The annotation contained a total of 129 genes, including 84 protein-coding genes, 37 tRNA genes and 8 rRNA genes. There were 3 genes using alternative initiation codons. Comparative genomics showed that the sequence of the Fagales species cp genome was relatively conserved, but there were still some high variation regions that could be used as molecular markers. The IR expansion event of B. platyphylla resulted in larger cp genomes and rps19 pseudogene formation. The simple sequence repeat (SSR) analysis showed that there were 105 SSRs in the cp genome of B. platyphylla. RNA editing sites recognition indicated that at least 80 RNA editing events occurred in the cp genome. Most of the substitutions were C to U, while a small proportion of them were not. In particular, three editing loci on the rRNA were converted to more than two other bases that had never been reported. For synonymous conversion, most of them increased the relative synonymous codon usage (RSCU) value of the codons. The phylogenetic analysis suggested that B. platyphylla had a closer evolutionary relationship with B. pendula than B. nana. CONCLUSIONS: In this study, we not only obtained and annotated the complete cp genome sequence of B. platyphylla, but we also identified new RNA editing sites and predicted the phylogenetic relationships among Fagales species. These findings will facilitate genomic, genetic engineering and phylogenetic studies of this important species.

Wang, W., et al. (2019). "PcMuORP1, an Oxathiapiprolin-Resistance Gene, Functions as a Novel Selection Marker for Phytophthora Transformation and CRISPR/Cas9 Mediated Genome Editing." Front Microbiol **10**: 2402.

Phytophthora, a genus of oomycetes, contains many devastating plant pathogens, which cause substantial economic losses worldwide. Recently, CRISPR/Cas9-based genome editing tool was introduced into Phytophthora to delineate the functionality of individual genes. The available selection markers for Phytophthora transformation, however, are limited, which can restrain transgenic manipulation in some cases. We hypothesized that PcMuORP1, an endogenous fungicide resistance gene from P. capsici that confers resistance to the fungicide oxathiapiprolin via an altered target site in the ORP1 protein, could be used as an alternative marker. To test this hypothesis, the gene PcMuORP1 was introduced into the CRISPR/Cas9 system and complementation of a deleted gene in P. capsici was achieved using it as a selection marker. All of the oxathiapiprolin-resistant transformants were confirmed to contain the marker gene, indicating that the positive screening rate was 100%. The novel selection marker could also be used in other representative Phytophthora species including P. sojae and P. litchii, also with 100% positive screening rate. Furthermore, comparative studies indicated that use of PcMuORP1 resulted in a much higher efficiency of screening compared to the conventional selection marker NPT II, especially in P. capsici. Successive subculture and asexual reproduction in the absence of selective pressure were found to result in the loss of the selection marker from the transformants, which indicates that the PcMuORP1 gene would have little long term influence on the fitness of transformants and could be reused as the selection marker in subsequent projects. Thus, we have created an alternative selection marker for Phytophthora transformation by using a fungicide resistance gene, which would accelerate functional studies of genes in these species.

Wang, X., et al. (2020). "CRISPR/Cas9 genome editing shows the important role of AZC_2928 gene in nitrogen-fixing bacteria of plants." Funct Integr Genomics **20**(5): 657-668.

AZC_2928 gene (GenBank accession no. BAF88926.1) of Azorhizobium caulinodans ORS571 has sequence homology to 2,3-aminomutases. However, its function is unknown. In this study, we are for the first time to knock out the gene completely in A. caulinodans ORS571 using the current advanced genome editing tool, CRISPR/Cas9. Our results show that the editing efficiency is 34% and AZC_2928 plays an extremely important role in regulating the formation of chemotaxis and biofilm. CRISPR/Cas9 knockout of AZC_2928 (big up tri, openAZC_2928) significantly enhanced chemotaxis and biofilm formation. Both chemotaxis and biofilm formation play an important role in nitrogen-fixing bacteria and their interaction with their host plants. Interestingly, AZC_2928 did not affect the motility of A. caulinodans ORS571 and the nodulation formation in their natural host plant,

Sesbania rostrata. Due to rhizobia needing to form bacteroids for symbiotic nitrogen fixation in mature nodules, AZC_2928 might have a direct influence on nitrogen fixation efficiency rather than the number of nodulations.

Weber, J., et al. (2017). "Functional Reconstitution of a Fungal Natural Product Gene Cluster by Advanced Genome Editing." ACS Synth Biol **6**(1): 62-68.

Filamentous fungi produce varieties of natural products even in a strain dependent manner. However, the genetic basis of chemical speciation between strains is still widely unknown. One example is trypacidin, a natural product of the opportunistic human pathogen Aspergillus fumigatus, which is not produced among different isolates. Combining computational analysis with targeted gene editing, we could link a single nucleotide insertion in the polyketide synthase of the trypacidin biosynthetic pathway and reconstitute its production in a nonproducing strain. Thus, we present a CRISPR/Cas9-based tool for advanced molecular genetic studies in filamentous fungi, exploiting selectable markers separated from the edited locus.

Weeks, D. P., et al. (2016). "Use of designer nucleases for targeted gene and genome editing in plants." Plant Biotechnol J **14**(2): 483-495.

The ability to efficiently inactivate or replace genes in model organisms allowed a rapid expansion of our understanding of many of the genetic, biochemical, molecular and cellular mechanisms that support life. With the advent of new techniques for manipulating genes and genomes that are applicable not only to single-celled organisms, but also to more complex organisms such as animals and plants, the speed with which scientists and biotechnologists can expand fundamental knowledge and apply that knowledge to improvements in medicine, industry and agriculture is set to expand in an exponential fashion. At the heart of these advancements will be the use of gene editing tools such as zinc finger nucleases, modified meganucleases, hybrid DNA/RNA oligonucleotides, TAL effector nucleases and modified CRISPR/Cas9. Each of these tools has the ability to precisely target one specific DNA sequence within a genome and (except for DNA/RNA oligonucleotides) to create a double-stranded DNA break. DNA repair to such breaks sometimes leads to gene knockouts or gene replacement by homologous recombination if exogenously supplied homologous DNA fragments are made available. Genome rearrangements are also possible to engineer. Creation and use of such genome rearrangements, gene knockouts and gene replacements by the plant science community is gaining significant momentum. To document some of this progress and to explore the technology's longer term potential, this review highlights present and future uses of designer nucleases to greatly expedite research with model plant systems and to engineer genes and genomes in major and minor crop species for enhanced food production.

Wu, S., et al. (2018). "Genome-wide association studies and CRISPR/Cas9-mediated gene editing identify regulatory variants influencing eyebrow thickness in humans." PLoS Genet **14**(9): e1007640.

Hair plays an important role in primates and is clearly subject to adaptive selection. While humans have lost most facial hair, eyebrows are a notable exception. Eyebrow thickness is heritable and widely believed to be subject to sexual selection. Nevertheless, few genomic studies have explored its genetic basis. Here, we performed a genome-wide scan for eyebrow thickness in 2961 Han Chinese. We identified two new loci of genome-wide significance, at 3q26.33 near SOX2 (rs1345417: P = 6.51x10(-10)) and at 5q13.2 near FOXD1 (rs12651896: P = 1.73x10(-8)). We further replicated our findings in the Uyghurs, a population from China characterized by East Asian-European admixture (N = 721), the CANDELA cohort from five Latin American countries (N = 2301), and the Rotterdam Study cohort of Dutch Europeans (N = 4411). A meta-analysis combining the full GWAS results from the three cohorts of full or partial Asian descent (Han Chinese, Uyghur and Latin Americans, N = 5983) highlighted a third signal of genome-wide significance at 2q12.3 (rs1866188: P = 5.81x10(-11)) near EDAR. We performed fine-mapping and prioritized four variants for further experimental verification. CRISPR/Cas9-mediated gene editing provided evidence that rs1345417 and rs12651896 affect the transcriptional activity of the nearby SOX2 and FOXD1 genes, which are both involved in hair development. Finally, suitable statistical analyses revealed that none of the associated variants showed clear signals of selection in any of the populations tested. Contrary to popular speculation, we found no evidence that eyebrow thickness is subject to strong selective pressure.

Xi, L., et al. (2015). "A novel two-step genome editing strategy with CRISPR-Cas9 provides new insights into telomerase action and TERT gene expression." Genome Biol **16**: 231.

BACKGROUND: To facilitate indefinite proliferation, stem cells and most cancer cells require the activity of telomerase, which counteracts the successive shortening of telomeres caused by incomplete DNA replication at the very end of each chromosome. Human telomerase activity is often determined by the expression level of telomerase reverse transcriptase (TERT), the catalytic subunit of the ribonucleoprotein complex. The low expression

level of TERT and the lack of adequate antibodies have made it difficult to study telomerase-related processes in human cells. RESULTS: To overcome the low CRISPR-Cas9 editing efficiency at the TERT locus, we develop a two-step "pop-in/pop-out" strategy to enrich cells that underwent homologous recombination (HR). Using this technique, we fuse an N-terminal FLAG-SNAP-tag to TERT, which allows us to reliably detect TERT in western blots, immunopurify it for biochemical analysis, and determine its subcellular localization by fluorescence microscopy. TERT co-localizes detectably with only 5-7 % of the telomeres at a time in S-phase HeLa cells; no nucleolar localization is detected. Furthermore, we extend this approach to perform single base-pair modifications in the TERT promoter; reverting a recurrent cancer-associated TERT promoter mutation in a urothelial cancer cell line results in decreased telomerase activity, indicating the mutation is causal for telomerase reactivation. CONCLUSIONS: We develop a two-step CRISPR-Cas9 genome editing strategy to introduce precise modifications at the endogenous TERT locus in human cell lines. This method provides a useful tool for studying telomerase biology, and suggests a general approach to edit loci with low targeting efficiency and to purify and visualize low abundance proteins.

Yamaguchi, T., et al. (2020). "Aspects of Gene Therapy Products Using Current Genome-Editing Technology in Japan." Hum Gene Ther **31**(19-20): 1043-1053.

The development of genome-editing technology could lead to breakthrough gene therapy. Genome editing has made it possible to easily knock out or modify a target gene, while current gene therapy using a virus vector or plasmid hampering modification with respect to gene replacement therapies. Clinical development using these genome-editing tools is progressing rapidly. However, it is also becoming clear that there is a possibility of unintended gene sequence modification or deletion, or the insertion of undesired genes, or the selection of cells with abnormalities in the cancer suppressor gene p53; these unwanted actions are not possible with current gene therapy. The Science Board of the Pharmaceuticals and Medical Devices Agency of Japan has compiled a report on the expected aspects of such genome-editing technology and the risks associated with it. This article summarizes the history of that discussion and compares the key concepts with information provided by other regulatory authorities.

Yang, H., et al. (2020). "Enhanced Transduction of Human Hematopoietic Stem Cells by AAV6 Vectors: Implications in Gene Therapy and Genome Editing." Mol Ther Nucleic Acids **20**: 451-458.

We have reported that of the 10 most commonly used adeno-associated virus (AAV) serotype vectors, AAV6 is the most efficient in transducing primary human hematopoietic stem cells (HSCs) in vitro, as well as in vivo. More recently, polyvinyl alcohol (PVA), was reported to be a superior replacement for human serum albumin (HSA) for ex vivo expansion of HSCs. Since HSA has been shown to increase the transduction efficiency of AAV serotype vectors, we evaluated whether PVA could also enhance the transduction efficiency of AAV6 vectors in primary human HSCs. We report here that up to 12-fold enhancement in the transduction efficiency of AAV6 vectors can be achieved in primary human HSCs with PVA. We also demonstrate that the improvement in the transduction efficiency is due to PVA-mediated improved entry and intracellular trafficking of AAV6 vectors in human hematopoietic cells in vitro, as well as in murine hepatocytes in vivo. Taken together, our studies suggest that the use of PVA is an attractive strategy to further improve the efficacy of AAV6 vectors. This has important implications in the optimal use of these vectors in the potential gene therapy and genome editing for human hemoglobinopathies such as beta-thalassemia and sickle cell disease.

Yang, J., et al. (2020). "Chromosome-level reference genome assembly and gene editing of the dead-leaf butterfly Kallima inachus." Mol Ecol Resour **20**(4): 1080-1092.

The leaf resemblance of Kallima (Nymphalidae) butterflies is an important ecological adaptive mechanism that increases their survival. However, the genetic mechanism underlying ecological adaptation remains unclear owing to a dearth of genomic information. Here, we determined the karyotype (n = 31) of the dead-leaf butterfly Kallima inachus, and generated a high-quality, chromosome-level assembly (568.92 Mb; contig N50: 19.20 Mb). We also identified candidate Z and W chromosomes. To our knowledge, this is the first study to report on these aspects of this species. In the assembled genome, 15,309 protein-coding genes and 49.86% repeat elements were annotated. Phylogenetic analysis showed that K. inachus diverged from Melitaea cinxia (no leaf resemblance), both of which are in Nymphalinae, around 40 million years ago. Demographic analysis indicated that the effective population size of K. inachus decreased during the last interglacial period in the Pleistocene. The wings of adults with the pigmentary gene ebony knocked out using CRISPR/Cas9 showed phenotypes in which the orange dorsal region and entire ventral surface darkened, suggesting its vital role in the ecological adaption of dead-leaf butterflies. Our results provide important genome resources for investigating the

genetic mechanism underlying protective resemblance in dead-leaf butterflies and insights into the molecular basis of protective coloration.

Yates, L. E., et al. (2019). "Glyco-recoded Escherichia coli: Recombineering-based genome editing of native polysaccharide biosynthesis gene clusters." Metab Eng **53**: 59-68.

Recombineering-based redesign of bacterial genomes by adding, removing or editing large segments of genomic DNA is emerging as a powerful technique for expanding the range of functions that an organism can perform. Here, we describe a glyco-recoding strategy whereby major non-essential polysaccharide gene clusters in K-12 Escherichia coli are replaced with orthogonal glycosylation components for both biosynthesis of heterologous glycan structures and site-specific glycan conjugation to target proteins. Specifically, the native enterobacterial common antigen (ECA) and O-polysaccharide (O-PS) antigen loci were systematically replaced with approximately 9-10 kbp of synthetic DNA encoding Campylobacter jejuni enzymes required for asparagine-linked (N-linked) protein glycosylation. Compared to E. coli cells carrying the same glycosylation machinery on extrachromosomal plasmids, glyco-recoded strains attached glycans to acceptor protein targets with equal or greater efficiency while exhibiting markedly better growth phenotypes and higher glycoprotein titers. Overall, our results define a convenient and reliable framework for bacterial glycome editing that provides a more stable route for chemical diversification of proteins in vivo and effectively expands the bacterial glycoengineering toolkit.

Yoshioka, I. and K. Kirimura (2021). "Rapid and marker-free gene replacement in citric acid-producing Aspergillus tubingensis (A. niger) WU-2223L by the CRISPR/Cas9 system-based genome editing technique using DNA fragments encoding sgRNAs." J Biosci Bioeng.

Strains belonging to Aspergillus section Nigri, including Aspergillus niger, are used for industrial production of citric acid from carbohydrates such as molasses and starch. The objective of this study was to construct the genome editing system that could enable rapid and efficient gene replacement in citric acid-producing fungi for genetic breeding. Using the citric acid-hyperproducer A. tubingensis (formerly A. niger) WU-2223L as a model strain, we developed a CRISPR/Cas9 system-based genome editing technique involving co-transformation of Cas9 and the DNA fragment encoding single guide RNA (sgRNA). Using this system, ATP-sulfurylase gene (sC) knock-out strain derived from WU-2223L was generated; the knock-out efficiency was 29 transformants when 5 mug Cas9 was added to 5 x 10(5) protoplasts. In the gene replacement method based on this system, a DNA fragment encoding sgRNAs that target both the gene of interest and marker gene was used, and replacement of nitrate reductase gene (niaD) using sC gene as a marker gene was attempted. More than 90% of the sC-knock-out transformants exhibited replaced niaD, indicating efficient gene replacement. Moreover, one-step marker rescue of the sC marker gene was accomplished by excising the knock-in donor via intramolecular homologous recombination, enabling marker-free genome editing and drastically shortening the gene replacement period by circumventing the transformation procedure to recover the sC gene. Thus, we succeeded in constructing a CRISPR/Cas9 system-based rapid and marker-free gene replacement system for the citric acid-hyperproducer strain WU-2223L.

Yusa, K. (2013). "Seamless genome editing in human pluripotent stem cells using custom endonuclease-based gene targeting and the piggyBac transposon." Nat Protoc **8**(10): 2061-2078.

I report here a detailed protocol for seamless genome editing using the piggyBac transposon in human pluripotent stem cells (hPSCs). Recent advances in custom endonucleases have enabled us to routinely perform genome editing in hPSCs. Conventional approaches use the Cre/loxP system that leaves behind residual sequences in the targeted genome. I used the piggyBac transposon to seamlessly remove a drug selection cassette and demonstrated safe genetic correction of a mutation causing alpha-1 antitrypsin deficiency in patient-derived hPSCs. An alternative approach to using the piggyBac transposon to correct mutations involves using single-stranded oligonucleotides, which is a faster process to complete. However, this experimental procedure is rather complicated and it may be hard to achieve homozygous modifications. In contrast, using the piggyBac transposon with drug selection-based enrichment of genetic modifications, as described here, is simple and can yield multiple correctly targeted clones, including homozygotes. Although two rounds of genetic manipulation are required to achieve homozygote modifications, the entire process takes approximately 3 months to complete.

Zafar, K., et al. (2020). "Precise CRISPR-Cas9 Mediated Genome Editing in Super Basmati Rice for Resistance Against Bacterial Blight by Targeting the Major Susceptibility Gene." Front Plant Sci **11**: 575.

Basmati rice is famous around the world for its flavor, aroma, and long grain. Its demand is increasing worldwide, especially in Asia. However, its production is threatened by various problems faced in the fields, resulting in major crop losses. One of the

major problems is bacterial blight caused by Xanthomonas oryzae pv. oryzae (Xoo). Xoo hijacks the host machinery by activating the susceptibility genes (OsSWEET family genes), using its endogenous transcription activator like effectors (TALEs). TALEs have effector binding elements (EBEs) in the promoter region of the OsSWEET genes. Out of six well-known TALEs found to have EBEs in Clade III SWEET genes, four are present in OsSWEET14 gene's promoter region. Thus, targeting the promoter of OsSWEET14 is very important for creating broad-spectrum resistance. To engineer resistance against bacterial blight, we established CRISPR-Cas9 mediated genome editing in Super Basmati rice by targeting 4 EBEs present in the promoter of OsSWEET14. We were able to obtain four different Super Basmati lines (SB-E1, SB-E2, SB-E3, and SB-E4) having edited EBEs of three TALEs (AvrXa7, PthXo3, and TalF). The edited lines were then evaluated in triplicate for resistance against bacterial blight by choosing one of the locally isolated virulent Xoo strains with AvrXa7 and infecting Super Basmati. The lines with deletions in EBE of AvrXa7 showed resistance against the Xoo strain. Thus, it was confirmed that edited EBEs provide resistance against their respective TALEs present in Xoo strains. In this study up to 9% editing efficiency was obtained. Our findings showed that CRISPR-Cas9 can be harnessed to generate resistance against bacterial blight in indigenous varieties, against locally prevalent Xoo strains.

Zaman, Q. U., et al. (2019). "CRISPR/Cas9-Mediated Multiplex Genome Editing of JAGGED Gene in Brassica napus L." Biomolecules **9**(11).

Pod shattering resistance is an essential component to achieving a high yield, which is a substantial objective in polyploid rapeseed cultivation. Previous studies have suggested that the Arabidopsis JAGGED (JAG) gene is a key factor implicated in the regulatory web of dehiscence fruit. However, its role in controlling pod shattering resistance in oilseed rape is still unknown. In this study, multiplex genome editing was carried out by the CRISPR/Cas9 system on five homoeologs (BnJAG.A02, BnJAG.C02, BnJAG.C06, BnJAG.A07, and BnJAG.A08) of the JAG gene. Knockout mutagenesis of all homoeologs drastically affected the development of the lateral organs in organizing pod shape and size. The cylindrical body of the pod comprised a number of undifferentiated cells like a callus, without distinctive valves, replum, septum, and valve margins. Pseudoseeds were produced, which were divided into two halves with an incomplete layer of cells (probably septum) that separated the undifferentiated cells. These mutants were not capable of generating any productive seeds for further generations. However, one mutant line was identified in which only a BnJAG.A08-NUB-Like paralog of the JAG gene was mutated. Knockout mutagenesis in BnJAG.A08-NUB gene caused significant changes in the pod dehiscence zone. The replum region of the mutant was increased to a great extent, resulting in enlarged cell size, bumpy fruit, and reduced length compared with the wild type. A higher replum-valve joint area may have increased the resistance to pod shattering by ~2-fold in JAG mutants compared with wild type. Our results offer a basis for understanding variations in Brassica napus fruit by mutating JAG genes and providing a way forward for other Brassicaceae species.

Zamyatnin, A. A., Jr. (2016). "Special Issue: Genome Editing and Gene Therapy." Biochemistry (Mosc) **81**(7): 651-652.

Gene therapy is one of the most rapidly developing fields of molecular medicine. Gene therapy allows simple transfer of genetic methods aimed at correcting pathological processes into clinical practice. However, a number of technical problems still exists limiting broad use of gene therapy approaches. This special issue discusses modern methods and approaches used for the development of novel, effective, and safe agents for gene therapy.

Zentner, G. E. and M. J. Wade (2017). "The promise and peril of CRISPR gene drives: Genetic variation and inbreeding may impede the propagation of gene drives based on the CRISPR genome editing technology." Bioessays **39**(10).

Gene drives are selfish genetic elements that use a variety of mechanisms to ensure they are transmitted to subsequent generations at greater than expected frequencies. Synthetic gene drives based on the clustered regularly interspersed palindromic repeats (CRISPR) genome editing system have been proposed as a way to alter the genetic characteristics of natural populations of organisms relevant to the goals of public health, conservation, and agriculture. Here, we review the principles and potential applications of CRISPR drives, as well as means proposed to prevent their uncontrolled spread. We also focus on recent work suggesting that factors such as natural genetic variation and inbreeding may represent substantial impediments to the propagation of CRISPR drives.

Zhang, L., et al. (2020). "Disruption or reduced expression of the orotidine-5'-decarboxylase gene pyrG increases citric acid production: a new discovery during recyclable genome editing in Aspergillus niger." Microb Cell Fact **19**(1): 76.

BACKGROUND: Aspergillus niger is a filamentous fungus used for the majority of global citric acid production. Recent developments in genome

editing now enable biotechnologists to engineer and optimize A. niger. Currently, however, genetic-leads for maximizing citric acid titers in industrial A. niger isolates is limited. RESULTS: In this study, we try to engineer two citric acid A. niger production isolates, WT-D and D353, to serve as platform strains for future high-throughput genome engineering. Consequently, we used genome editing to simultaneously disrupt genes encoding the orotidine-5'-decarboxylase (pyrG) and non-homologous end-joining component (kusA) to enable use of the pyrG selection/counter selection system, and to elevate homologous recombination rates, respectively. During routine screening of these pyrG mutant strains, we unexpectedly observed a 2.17-fold increase in citric acid production when compared to the progenitor controls, indicating that inhibition of uridine/pyrimidine synthesis may increase citric acid titers. In order to further test this hypothesis, the pyrG gene was placed under the control of a tetracycline titratable cassette, which confirmed that reduced expression of this gene elevated citric acid titers in both shake flask and bioreactor fermentation. Subsequently, we conducted intracellular metabolomics analysis, which demonstrated that pyrG disruption enhanced the glycolysis flux and significantly improved abundance of citrate and its precursors. CONCLUSIONS: In this study, we deliver two citric acid producing isolates which are amenable to high throughput genetic manipulation due to pyrG/kusA deletion. Strikingly, we demonstrate for the first time that A. niger pyrG is a promising genetic lead for generating citric acid hyper-producing strains. Our data support the hypothesis that uridine/pyrimidine biosynthetic pathway offer future avenues for strain engineering efforts.

Zhang, Y., et al. (2021). "CRISPR-Cas9 multiplex genome editing of the hydroxyproline-O-galactosyltransferase gene family alters arabinogalactan-protein glycosylation and function in Arabidopsis." BMC Plant Biol **21**(1): 16.

BACKGROUND: Arabinogalactan-proteins (AGPs) are a class of hydroxyproline-rich proteins (HRGPs) that are heavily glycosylated (> 90%) with type II arabinogalactans (AGs). AGPs are implicated in various plant growth and development processes including cell expansion, somatic embryogenesis, root and stem growth, salt tolerance, hormone signaling, male and female gametophyte development, and defense. To date, eight Hyp-O-galactosyltransferases (GALT2-6, HPGT1-3) have been identified; these enzymes are responsible for adding the first sugar, galactose, onto AGPs. Due to gene redundancy among the GALTs, single or double galt genetic knockout mutants are often not sufficient to fully reveal their biological functions. RESULTS: Here, we report the successful application of CRISPR-Cas9 gene editing/multiplexing technology to generate higher-order knockout mutants of five members of the GALT gene family (GALT2-6). AGPs analysis of higher-order galt mutants (galt2 galt5, galt3 galt4 galt6, and galt2 galt3 galt4 galt5 gal6) demonstrated significantly less glycosylated AGPs in rosette leaves, stems, and siliques compared to the corresponding wild-type organs. Monosaccharide composition analysis of AGPs isolated from rosette leaves revealed significant decreases in arabinose and galactose in all the higher-order galt mutants. Phenotypic analyses revealed that mutation of two or more GALT genes was able to overcome the growth inhibitory effect of beta-D-Gal-Yariv reagent, which specifically binds to beta-1,3-galactan backbones on AGPs. In addition, the galt2 galt3 galt4 galt5 gal6 mutant exhibited reduced overall growth, impaired root growth, abnormal pollen, shorter siliques, and reduced seed set. Reciprocal crossing experiments demonstrated that galt2 galt3 galt4 galt5 gal6 mutants had defects in the female gametophyte which were responsible for reduced seed set. CONCLUSIONS: Our CRISPR/Cas9 gene editing/multiplexing approach provides a simpler and faster way to generate higher-order mutants for functional characterization compared to conventional genetic crossing of T-DNA mutant lines. Higher-order galt mutants produced and characterized in this study provide insight into the relationship between sugar decorations and the various biological functions attributed to AGPs in plants.

Zhang, Y., et al. (2018). "Application of CRISPR/Cas9 Gene Editing System on MDV-1 Genome for the Study of Gene Function." Viruses **10**(6).

Marek's disease virus (MDV) is a member of alphaherpesviruses associated with Marek's disease, a highly contagious neoplastic disease in chickens. Complete sequencing of the viral genome and recombineering techniques using infectious bacterial artificial chromosome (BAC) clones of Marek's disease virus genome have identified major genes that are associated with pathogenicity. Recent advances in CRISPR/Cas9-based gene editing have given opportunities for precise editing of the viral genome for identifying pathogenic determinants. Here we describe the application of CRISPR/Cas9 gene editing approaches to delete the Meq and pp38 genes from the CVI988 vaccine strain of MDV. This powerful technology will speed up the MDV gene function studies significantly, leading to a better understanding of the molecular mechanisms of MDV pathogenesis.

Zhang, Z. Y., et al. (2020). "Gene therapy and genome editing for primary immunodeficiency diseases." Genes Dis **7**(1): 38-51.

In past two decades the gene therapy using genetic modified autologous hematopoietic stem cells (HSCs) transduced with the viral vector has become a promising alternative option for treating primary immunodeficiency diseases (PIDs). Despite of some pitfalls at early stage clinical trials, the field of gene therapy has advanced significantly in the last decade with improvements in viral vector safety, preparatory regime for manufacturing high quality virus, automated CD34 cell purification. Hence, the overall outcome from the clinical trials for the different PIDs has been very encouraging. In addition to the viral vector based gene therapy, the recent fast moving forward developments in genome editing using engineered nucleases in HSCs has provided a new promising platform for the treatment of PIDs. This review provides an overall outcome and progress in gene therapy clinical trials for SCID-X, ADA-SCID, WAS, X- CGD, and the recent developments in genome editing technology applied in HSCs for developing potential therapy, particular in the key studies for PIDs.

Zheng, W., et al. (2020). "Establishment of recombineering genome editing system in Paraburkholderia megapolitana empowers activation of silent biosynthetic gene clusters." Microb Biotechnol **13**(2): 397-405.

The Burkholderiales are an emerging source of bioactive natural products. Their genomes contain a large number of cryptic biosynthetic gene clusters (BGCs), indicating great potential for novel structures. However, the lack of genetic tools for the most of Burkholderiales strains restricts the mining of these cryptic BGCs. We previously discovered novel phage recombinases Redalphabeta7029 from Burkholderiales strain DSM 7029 that could help in efficiently editing several Burkholderiales genomes and established the recombineering genome editing system in Burkholderialse species. Herein, we report the application of this phage recombinase system in another species Paraburkholderia megapolitana DSM 23488, resulting in activation of two silent non-ribosomal peptide synthetase/polyketide synthase BGCs. A novel class of lipopeptide, haereomegapolitanin, was identified through spectroscopic characterization. Haereomegapolitanin A represents an unusual threonine-tagged lipopeptide which is longer than the predicted NRPS assembly line. This recombineering-mediated genome editing system shows great potential for genetic manipulation of more Burkholderiales species to activate silent BGCs for bioactive metabolites discovery.

Zhou, X. C. and Y. Z. Xing (2016). "[The application of genome editing in identification of plant gene function and crop breeding]." Yi Chuan **38**(3): 227-242.

Plant genome can be modified via current biotechnology with high specificity and excellent efficiency. Zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN) and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated 9 (Cas9) system are the key engineered nucleases used in the genome editing. Genome editing techniques enable gene targeted mutagenesis, gene knock-out, gene insertion or replacement at the target sites during the endogenous DNA repair process, including non-homologous end joining (NHEJ) and homologous recombination (HR), triggered by the induction of DNA double-strand break (DSB). Genome editing has been successfully applied in the genome modification of diverse plant species, such as Arabidopsis thaliana, Oryza sativa, and Nicotiana tabacum. In this review, we summarize the application of genome editing in identification of plant gene function and crop breeding. Moreover, we also discuss the improving points of genome editing in crop precision genetic improvement for further study.

Zhu, C., et al. (2019). "Genome sequencing and CRISPR/Cas9 gene editing of an early flowering Mini-Citrus (Fortunella hindsii)." Plant Biotechnol J **17**(11): 2199-2210.

Hongkong kumquat (Fortunella hindsii) is a wild citrus species characterized by dwarf plant height and early flowering. Here, we identified the monoembryonic F. hindsii (designated as 'Mini-Citrus') for the first time and constructed its selfing lines. This germplasm constitutes an ideal model for the genetic and functional genomics studies of citrus, which have been severely hindered by the long juvenility and inherent apomixes of citrus. F. hindsii showed a very short juvenile period (~8 months) and stable monoembryonic phenotype under cultivation. We report the first de novo assembled 373.6 Mb genome sequences (Contig-N50 2.2 Mb and Scaffold-N50 5.2 Mb) for F. hindsii. In total, 32 257 protein-coding genes were annotated, 96.9% of which had homologues in other eight Citrinae species. The phylogenomic analysis revealed a close relationship of F. hindsii with cultivated citrus varieties, especially with mandarin. Furthermore, the CRISPR/Cas9 system was demonstrated to be an efficient strategy to generate target mutagenesis on F. hindsii. The modifications of target genes in the CRISPR-modified F. hindsii were predominantly 1-bp insertions or small deletions. This genetic transformation system based on F. hindsii could shorten the whole process from explant to T1 mutant to about 15 months. Overall, due to its short juvenility, monoembryony, close genetic background to cultivated citrus and applicability of CRISPR, F. hindsii shows

unprecedented potentials to be used as a model species for citrus research.

Zuo, Q., et al. (2016). "Site-Directed Genome Knockout in Chicken Cell Line and Embryos Can Use CRISPR/Cas Gene Editing Technology." G3 (Bethesda) **6**(6): 1787-1792.

The present study established an efficient genome editing approach for the construction of stable transgenic cell lines of the domestic chicken (Gallus gallus domesticus). Our objectives were to facilitate the breeding of high-yield, high-quality chicken strains, and to investigate gene function in chicken stem cells. Three guide RNA (gRNAs) were designed to knockout the C2EIP gene, and knockout efficiency was evaluated in DF-1 chicken fibroblasts and chicken ESCs using the luciferase single-strand annealing (SSA) recombination assay, T7 endonuclease I (T7EI) assay, and TA clone sequencing. In addition, the polyethylenimine-encapsulated Cas9/gRNA plasmid was injected into fresh fertilized eggs. At 4.5 d later, frozen sections of the embryos were prepared, and knockout efficiency was evaluated by the T7EI assay. SSA assay results showed that luciferase activity of the vector expressing gRNA-3 was double that of the control. Results of the T7EI assay and TA clone sequencing indicated that Cas9/gRNA vector-mediated gene knockdown efficiency was approximately 27% in both DF-1 cells and ESCs. The CRISPR/Cas9 vector was also expressed in chicken embryos, resulting in gene knockdown in three of the 20 embryos (gene knockdown efficiency 15%). Taken together, our results indicate that the CRISPR/Cas9 system can mediate stable gene knockdown at the cell and embryo levels in domestic chickens.

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5/22/2021