# Stem Cell and Genome editing Research Literatures

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**Abstract**: Stem cells are derived from embryonic and non-embryonic tissues. Most stem cell studies are for animal stem cells and plants have also stem cell. Stem cells were discovered in 1981 from early mouse embryos. Stem cells have the potential to develop into all different cell types in the living body. Stem cell is a body repair system. When a stem cell divides it can be still a stem cell or become adult cell, such as a brain cell. Stem cells are unspecialized cells and can renew themselves by cell division, and stem cells can also differentiate to adult cells with special functions. Stem cells replace the old cells and repair the damaged tissues. Embryonic stem cells can become all cell types of the body because they are pluripotent. Adult stem cells are thought to be limited to differentiating into different cell types of their tissue of origin. This article introduces recent research reports as references in the related studies. Genome editing, or genome engineering is a type of genetic engineering in which DNA is inserted, deleted, modified or replaced in the genome of a living organism. In 2018, the common methods for such editing use 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. 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. Nine genome editors were available as of 2017.

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**Key words**: stem cell; genome editing; life; research; literature

## Introduction

Stem cells are derived from embryonic and non-embryonic tissues. Most stem cell studies are for animal stem cells and plants have also stem cell. Stem cells were discovered in 1981 from early mouse embryos. Stem cells have the potential to develop into all different cell types in the living body. Stem cell is a body repair system. When a stem cell divides it can be still a stem cell or become adult cell, such as a brain cell. Stem cells are unspecialized cells and can renew themselves by cell division, and stem cells can also differentiate to adult cells with special functions. Stem cells replace the old cells and repair the damaged tissues. Embryonic stem cells can become all cell types of the body because they are pluripotent. Adult stem cells are thought to be limited to differentiating into different cell types of their tissue of origin. This article introduces recent research reports as references in the related studies. **Genome editing**, or **genome engineering** is a type of genetic engineering in which DNA is inserted, deleted, modified or replaced in the genome of a living organism. In 2018, the common methods for such editing use 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. 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. Nine genome editors were available as of 2017.

The following introduces recent reports as references in the related studies.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

Vanden Bempt, M., et al. (2016). "Generation of the Fip1l1-Pdgfra fusion gene using CRISPR/Cas genome editing." Leukemia **30**(9): 1913-1916.

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, 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.

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.

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.

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.

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.

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.

The above contents are the collected information from Internet and public resources to offer to the people for the convenient reading and information disseminating and sharing.

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