Genome editing in Human and Stem Cell 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.

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

Introduction

The stem cell is the origin of an organism's life that has the potential to develop into many different types of cells in life bodies. In many tissues stem cells serve as a sort of internal repair system, dividing essentially without limit to replenish other cells as long as the person or animal is still alive. When a stem cell divides, each new cell has the potential either to remain a stem cell or become another type of cell with a more specialized function, such as a red blood cell or a brain cell. This article introduces recent research reports as references in the related studies.

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

Bahal, R., et al. (2016). "In vivo correction of anaemia in beta-thalassemic mice by gammaPNA-mediated gene editing with nanoparticle delivery." Nat Commun **7**: 13304.

The blood disorder, beta-thalassaemia, is considered an attractive target for gene correction. Site-specific triplex formation has been shown to induce DNA repair and thereby catalyse genome editing. Here we report that triplex-forming peptide nucleic acids (PNAs) substituted at the gamma position plus stimulation of the stem cell factor (SCF)/c-Kit pathway yielded high levels of gene editing in haematopoietic stem cells (HSCs) in a mouse model of human beta-thalassaemia. Injection of thalassemic mice with SCF plus nanoparticles containing gammaPNAs and donor DNAs ameliorated the disease phenotype, with sustained elevation of blood haemoglobin levels into the normal range, reduced reticulocytosis, reversal of splenomegaly and up to 7% beta-globin gene correction in HSCs, with extremely low off-target effects. The combination of nanoparticle delivery, next generation gammaPNAs and SCF treatment may offer a minimally invasive treatment for genetic disorders of the blood that can be achieved safely and simply by intravenous administration.

Bak, R. O. and M. H. Porteus (2017). "CRISPR-Mediated Integration of Large Gene Cassettes Using AAV Donor Vectors." Cell Rep **20**(3): 750-756.

The CRISPR/Cas9 system has recently been shown to facilitate high levels of precise genome editing using adeno-associated viral (AAV) vectors to serve as donor template DNA during homologous recombination (HR). However, the maximum AAV packaging capacity of approximately 4.5 kb limits the donor size. Here, we overcome this constraint by showing that two co-transduced AAV vectors can serve as donors during consecutive HR events for the integration of large transgenes. Importantly, the method involves a single-step procedure applicable to primary cells with relevance to therapeutic genome editing. We use the methodology in primary human T cells and CD34(+) hematopoietic stem and progenitor cells to site-specifically integrate an expression cassette that, as a single donor vector, would otherwise amount to a total of 6.5 kb. This approach now provides an efficient way to integrate large transgene cassettes into the genomes of primary human cells using HR-mediated genome editing with AAV vectors.

Bertero, A., et al. (2018). "Conditional Manipulation of Gene Function in Human Cells with Optimized Inducible shRNA." Curr Protoc Stem Cell Biol **44**: 5C 4 1-5C 4 48.

The difficulties involved in conditionally perturbing complex gene expression networks represent major challenges toward defining the mechanisms controlling human development, physiology, and disease. We developed an OPTimized inducible KnockDown (OPTiKD) platform that addresses the limitations of previous approaches by allowing streamlined, tightly-controlled, and potent loss-of-function experiments for both single and multiple genes. The method relies on single-step genetic engineering of the AAVS1 genomic safe harbor with an optimized tetracycline-responsive cassette driving one or more inducible short hairpin RNAs (shRNAs). OPTiKD provides homogeneous, dose-responsive, and reversible gene knockdown. When implemented in human pluripotent stem cells (hPSCs), the approach can be then applied to a broad range of hPSC-derived mature cell lineages that include neurons, cardiomyocytes, and hepatocytes. Generation of OPTiKD hPSCs in commonly used culture conditions is simple (plasmid based), rapid (two weeks), and highly efficient (>95%). Overall, this method facilitates the functional annotation of the human genome in health and disease. (c) 2018 by John Wiley & Sons, Inc.

Bressan, R. B., et al. (2017). "Efficient CRISPR/Cas9-assisted gene targeting enables rapid and precise genetic manipulation of mammalian neural stem cells." Development **144**(4): 635-648.

Mammalian neural stem cell (NSC) lines provide a tractable model for discovery across stem cell and developmental biology, regenerative medicine and neuroscience. They can be derived from foetal or adult germinal tissues and continuously propagated in vitro as adherent monolayers. NSCs are clonally expandable, genetically stable, and easily transfectable - experimental attributes compatible with targeted genetic manipulations. However, gene targeting, which is crucial for functional studies of embryonic stem cells, has not been exploited to date in NSC lines. Here, we deploy CRISPR/Cas9 technology to demonstrate a variety of sophisticated genetic modifications via gene targeting in both mouse and human NSC lines, including: (1) efficient targeted transgene insertion at safe harbour loci (Rosa26 and AAVS1); (2) biallelic knockout of neurodevelopmental transcription factor genes; (3) simple knock-in of epitope tags and fluorescent reporters (e.g. Sox2-V5 and Sox2-mCherry); and (4) engineering of glioma mutations (TP53 deletion; H3F3A point mutations). These resources and optimised methods enable facile and scalable genome editing in mammalian NSCs, providing significant new opportunities for functional genetic analysis.

Brookhouser, N., et al. (2017). "May I Cut in? Gene Editing Approaches in Human Induced Pluripotent Stem Cells." Cells **6**(1).

In the decade since Yamanaka and colleagues described methods to reprogram somatic cells into a pluripotent state, human induced pluripotent stem cells (hiPSCs) have demonstrated tremendous promise in numerous disease modeling, drug discovery, and regenerative medicine applications. More recently, the development and refinement of advanced gene transduction and editing technologies have further accelerated the potential of hiPSCs. In this review, we discuss the various gene editing technologies that are being implemented with hiPSCs. Specifically, we describe the emergence of technologies including zinc-finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 that can be used to edit the genome at precise locations, and discuss the strengths and weaknesses of each of these technologies. In addition, we present the current applications of these technologies in elucidating the mechanisms of human development and disease, developing novel and effective therapeutic molecules, and engineering cell-based therapies. Finally, we discuss the emerging technological advances in targeted gene editing methods.

Brunetti, L., et al. (2018). "Highly Efficient Gene Disruption of Murine and Human Hematopoietic Progenitor Cells by CRISPR/Cas9." J Vis Exp (134).

Advances in the hematopoietic stem cell (HSCs) field have been aided by methods to genetically engineer primary progenitor cells as well as animal models. Complete gene ablation in HSCs required the generation of knockout mice from which HSCs could be isolated, and gene ablation in primary human HSCs was not possible. Viral transduction could be used for knock-down approaches, but these suffered from variable efficacy. In general, genetic manipulation of human and mouse hematopoietic cells was hampered by low efficiencies and extensive time and cost commitments. Recently, CRISPR/Cas9 has dramatically expanded the ability to engineer the DNA of mammalian cells. However, the application of CRISPR/Cas9 to hematopoietic cells has been challenging, mainly due to their low transfection efficiencies, the toxicity of plasmid-based approaches and the slow turnaround time of virus-based protocols. A rapid method to perform CRISPR/Cas9-mediated gene editing in murine and human hematopoietic stem and progenitor cells with knockout efficiencies of up to 90% is provided in this article. This approach utilizes a ribonucleoprotein (RNP) delivery strategy with a streamlined three-day workflow. The use of Cas9-sgRNA RNP allows for a hit-and-run approach,

introducing no exogenous DNA sequences in the genome of edited cells and reducing off-target effects. The RNP-based method is fast and straightforward: it does not require cloning of sgRNAs, virus preparation or specific sgRNA chemical modification. With this protocol, scientists should be able to successfully generate knockouts of a gene of interest in primary hematopoietic cells within a week, including downtimes for oligonucleotide synthesis. This approach will allow a much broader group of users to adapt this protocol for their needs.

Burnight, E. R., et al. (2017). "Using CRISPR-Cas9 to Generate Gene-Corrected Autologous iPSCs for the Treatment of Inherited Retinal Degeneration." Mol Ther **25**(9): 1999-2013.

Patient-derived induced pluripotent stem cells (iPSCs) hold great promise for autologous cell replacement. However, for many inherited diseases, treatment will likely require genetic repair pre-transplantation. Genome editing technologies are useful for this application. The purpose of this study was to develop CRISPR-Cas9-mediated genome editing strategies to target and correct the three most common types of disease-causing variants in patient-derived iPSCs: (1) exonic, (2) deep intronic, and (3) dominant gain of function. We developed a homology-directed repair strategy targeting a homozygous Alu insertion in exon 9 of male germ cell-associated kinase (MAK) and demonstrated restoration of the retinal transcript and protein in patient cells. We generated a CRISPR-Cas9-mediated non-homologous end joining (NHEJ) approach to excise a major contributor to Leber congenital amaurosis, the IVS26 cryptic-splice mutation in CEP290, and demonstrated correction of the transcript and protein in patient iPSCs. Lastly, we designed allele-specific CRISPR guides that selectively target the mutant Pro23His rhodopsin (RHO) allele, which, following delivery to both patient iPSCs in vitro and pig retina in vivo, created a frameshift and premature stop that would prevent transcription of the disease-causing variant. The strategies developed in this study will prove useful for correcting a wide range of genetic variants in genes that cause inherited retinal degeneration.

Byrne, S. M. and G. M. Church (2015). "Crispr-mediated Gene Targeting of Human Induced Pluripotent Stem Cells." Curr Protoc Stem Cell Biol **35**: 5A 8 1-22.

CRISPR/Cas9 nuclease systems can create double-stranded DNA breaks at specific sequences to efficiently and precisely disrupt, excise, mutate, insert, or replace genes. However, human embryonic stem or induced pluripotent stem cells (iPSCs) are more difficult to transfect and less resilient to DNA damage than immortalized tumor cell lines. Here, we describe an optimized protocol for genome engineering of human iPSCs using a simple transient transfection of plasmids and/or single-stranded oligonucleotides. With this protocol, we achieve transfection efficiencies greater than 60%, with gene disruption efficiencies from 1-25% and gene insertion/replacement efficiencies from 0.5-10% without any further selection or enrichment steps. We also describe how to design and assess optimal sgRNA target sites and donor targeting vectors; cloning individual iPSC by single cell FACS sorting, and genotyping successfully edited cells.

Cai, L., et al. (2018). "A Universal Approach to Correct Various HBB Gene Mutations in Human Stem Cells for Gene Therapy of Beta-Thalassemia and Sickle Cell Disease." Stem Cells Transl Med **7**(1): 87-97.

Beta-thalassemia is one of the most common recessive genetic diseases, caused by mutations in the HBB gene. Over 200 different types of mutations in the HBB gene containing three exons have been identified in patients with beta-thalassemia (beta-thal) whereas a homozygous mutation in exon 1 causes sickle cell disease (SCD). Novel therapeutic strategies to permanently correct the HBB mutation in stem cells that are able to expand and differentiate into erythrocytes producing corrected HBB proteins are highly desirable. Genome editing aided by CRISPR/Cas9 and other site-specific engineered nucleases offers promise to precisely correct a genetic mutation in the native genome without alterations in other parts of the human genome. Although making a sequence-specific nuclease to enhance correction of a specific HBB mutation by homology-directed repair (HDR) is becoming straightforward, targeting various HBB mutations of beta-thal is still challenging because individual guide RNA as well as a donor DNA template for HDR of each type of HBB gene mutation have to be selected and validated. Using human induced pluripotent stem cells (iPSCs) from two beta-thal patients with different HBB gene mutations, we devised and tested a universal strategy to achieve targeted insertion of the HBB cDNA in exon 1 of HBB gene using Cas9 and two validated guide RNAs. We observed that HBB protein production was restored in erythrocytes derived from iPSCs of two patients. This strategy of restoring functional HBB gene expression will be able to correct most types of HBB gene mutations in beta-thal and SCD. Stem Cells Translational Medicine 2018;7:87-97.

Carlson-Stevermer, J., et al. (2017). "Assembly of CRISPR ribonucleoproteins with biotinylated

oligonucleotides via an RNA aptamer for precise gene editing." Nat Commun **8**(1): 1711.

Writing specific DNA sequences into the human genome is challenging with non-viral gene-editing reagents, since most of the edited sequences contain various imprecise insertions or deletions. We developed a modular RNA aptamer-streptavidin strategy, termed S1mplex, to complex CRISPR-Cas9 ribonucleoproteins with a nucleic acid donor template, as well as other biotinylated molecules such as quantum dots. In human cells, tailored S1mplexes increase the ratio of precisely edited to imprecisely edited alleles up to 18-fold higher than standard gene-editing methods, and enrich cell populations containing multiplexed precise edits up to 42-fold. These advances with versatile, preassembled reagents could greatly reduce the time and cost of in vitro or ex vivo gene-editing applications in precision medicine and drug discovery and aid in the development of increased and serial dosing regimens for somatic gene editing in vivo.

Carlson-Stevermer, J., et al. (2016). "High-Content Analysis of CRISPR-Cas9 Gene-Edited Human Embryonic Stem Cells." Stem Cell Reports **6**(1): 109-120.

CRISPR-Cas9 gene editing of human cells and tissues holds much promise to advance medicine and biology, but standard editing methods require weeks to months of reagent preparation and selection where much or all of the initial edited samples are destroyed during analysis. ArrayEdit, a simple approach utilizing surface-modified multiwell plates containing one-pot transcribed single-guide RNAs, separates thousands of edited cell populations for automated, live, high-content imaging and analysis. The approach lowers the time and cost of gene editing and produces edited human embryonic stem cells at high efficiencies. Edited genes can be expressed in both pluripotent stem cells and differentiated cells. This preclinical platform adds important capabilities to observe editing and selection in situ within complex structures generated by human cells, ultimately enabling optical and other molecular perturbations in the editing workflow that could refine the specificity and versatility of gene editing.

Cavazzana, M., et al. (2017). "Gene Therapy for beta-Hemoglobinopathies." Mol Ther **25**(5): 1142-1154.

beta-Thalassemia and sickle cell disease (SCD) are the world's two most widely disseminated hereditary hemoglobinopathies. beta-Thalassemia originated in the Mediterranean, Middle Eastern, and Asian regions, and SCD originated in central Africa. However, subsequent population migration means that these two diseases are now global and thus constitute a growing health problem in many countries. Despite remarkable improvements in medical care for patients with beta-hemoglobinopathies, there is still only one definitive treatment option: allogeneic hematopoietic stem cell (HSC) transplantation. The development of gene therapy for beta-hemoglobinopathies has been justified by (1) the limited availability of human leukocyte antigen (HLA)-identical donors, (2) the narrow window of application of HSC transplantation to the youngest patients, and (3) recent advances in HSC-based gene therapy. The huge ongoing efforts in translational medicine and the high number of related publications show that gene therapy has the potential to become the treatment of choice for patients who lack either an HLA genoidentical sibling or an alternative, medically acceptable donor. In this dynamic scientific context, we first summarize the main steps toward clinical translation of this therapeutic approach and then discuss novel lentiviral- and genome editing-based treatment strategies for beta-hemoglobinopathies.

Chang, K. H., et al. (2017). "Long-Term Engraftment and Fetal Globin Induction upon BCL11A Gene Editing in Bone-Marrow-Derived CD34(+) Hematopoietic Stem and Progenitor Cells." Mol Ther Methods Clin Dev **4**: 137-148.

To develop an effective and sustainable cell therapy for sickle cell disease (SCD), we investigated the feasibility of targeted disruption of the BCL11A gene, either within exon 2 or at the GATAA motif in the intronic erythroid-specific enhancer, using zinc finger nucleases in human bone marrow (BM) CD34(+) hematopoietic stem and progenitor cells (HSPCs). Both targeting strategies upregulated fetal globin expression in erythroid cells to levels predicted to inhibit hemoglobin S polymerization. However, complete inactivation of BCL11A resulting from bi-allelic frameshift mutations in BCL11A exon 2 adversely affected erythroid enucleation. In contrast, bi-allelic disruption of the GATAA motif in the erythroid enhancer of BCL11A did not negatively impact enucleation. Furthermore, BCL11A exon 2-edited BM-CD34(+) cells demonstrated a significantly reduced engraftment potential in immunodeficient mice. Such an adverse effect on HSPC function was not observed upon BCL11A erythroid-enhancer GATAA motif editing, because enhancer-edited CD34(+) cells achieved robust long-term engraftment and gave rise to erythroid cells with elevated levels of fetal globin expression when chimeric BM was cultured ex vivo. Altogether, our results support further clinical development of the BCL11A erythroid-specific enhancer editing in BM-CD34(+) HSPCs as an autologous stem cell therapy in SCD patients.

Charlesworth, C. T., et al. (2018). "Priming Human Repopulating Hematopoietic Stem and Progenitor Cells for Cas9/sgRNA Gene Targeting." Mol Ther Nucleic Acids **12**: 89-104.

Engineered nuclease-mediated gene targeting through homologous recombination (HR) in hematopoietic stem and progenitor cells (HSPCs) has the potential to treat a variety of genetic hematologic and immunologic disorders. Here, we identify critical parameters to reproducibly achieve high frequencies of RNA-guided (single-guide RNA [sgRNA]; CRISPR)-Cas9 nuclease (Cas9/sgRNA) and rAAV6-mediated HR at the beta-globin (HBB) locus in HSPCs. We identified that by transducing HSPCs with rAAV6 post-electroporation, there was a greater than 2-fold electroporation-aided transduction (EAT) of rAAV6 endocytosis with roughly 70% of the cell population having undergone transduction within 2 hr. When HSPCs are cultured at low densities (1 x 10(5) cells/mL) prior to HBB targeting, HSPC expansion rates are significantly positively correlated with HR frequencies in vitro as well as in repopulating cells in immunodeficient NSG mice in vivo. We also show that culturing fluorescence-activated cell sorting (FACS)-enriched HBB-targeted HSPCs at low cell densities in the presence of the small molecules, UM171 and SR1, stimulates the expansion of gene-edited HSPCs as measured by higher engraftment levels in immunodeficient mice. This work serves not only as an optimized protocol for genome editing HSPCs at the HBB locus for the treatment of beta-hemoglobinopathies but also as a foundation for editing HSPCs at other loci for both basic and translational research.

Chen, Y., et al. (2015). "Engineering Human Stem Cell Lines with Inducible Gene Knockout using CRISPR/Cas9." Cell Stem Cell **17**(2): 233-244.

Precise temporal control of gene expression or deletion is critical for elucidating gene function in biological systems. However, the establishment of human pluripotent stem cell (hPSC) lines with inducible gene knockout (iKO) remains challenging. We explored building iKO hPSC lines by combining CRISPR/Cas9-mediated genome editing with the Flp/FRT and Cre/LoxP system. We found that "dual-sgRNA targeting" is essential for biallelic knockin of FRT sequences to flank the exon. We further developed a strategy to simultaneously insert an activity-controllable recombinase-expressing cassette and remove the drug-resistance gene, thus speeding up the generation of iKO hPSC lines. This two-step strategy was used to establish human embryonic stem cell (hESC) and induced pluripotent stem cell (iPSC) lines with iKO of SOX2, PAX6, OTX2, and AGO2, genes that exhibit diverse structural layout and temporal expression patterns. The availability of iKO hPSC lines will substantially transform the way we examine gene function in human cells.

Chen, Y. H. and S. M. Pruett-Miller (2018). "Improving single-cell cloning workflow for gene editing in human pluripotent stem cells." Stem Cell Res **31**: 186-192.

The availability of human pluripotent stem cells (hPSCs) and progress in genome engineering technology have altered the way we approach scientific research and drug development screens. Unfortunately, the procedures for genome editing of hPSCs often subject cells to harsh conditions that compromise viability: a major problem that is compounded by the innate challenge of single-cell culture. Here we describe a generally applicable workflow that supports single-cell cloning and expansion of hPSCs after genome editing and single-cell sorting. Stem-Flex and RevitaCell supplement, in combination with Geltrex or Vitronectin (VN), promote reliable single-cell growth in a feeder-free and defined environment. Characterization of final genome-edited clones reveals that pluripotency and normal karyotype are retained following this single-cell culture protocol. This time-efficient and simplified culture method paves the way for high-throughput hPSC culture and will be valuable for both basic research and clinical applications.

Cornu, T. I., et al. (2015). "Editing CCR5: a novel approach to HIV gene therapy." Adv Exp Med Biol **848**: 117-130.

Acquired immunodeficiency syndrome (AIDS) is a life-threatening disorder caused by infection of individuals with the human immunodeficiency virus (HIV). Entry of HIV-1 into target cells depends on the presence of two surface proteins on the cell membrane: CD4, which serves as the main receptor, and either CCR5 or CXCR4 as a co-receptor. A limited number of people harbor a genomic 32-bp deletion in the CCR5 gene (CCR532), leading to expression of a truncated gene product that provides resistance to HIV-1 infection in individuals homozygous for this mutation. Moreover, allogeneic hematopoietic stem cell (HSC) transplantation with CCR532 donor cells seems to confer HIV-1 resistance to the recipient as well. However, since Delta32 donors are scarce and allogeneic HSC transplantation is not exempt from risks, the development of gene editing tools to knockout CCR5 in the genome of autologous cells is highly warranted. Targeted gene editing can be accomplished with designer nucleases, which essentially are engineered restriction enzymes that can be designed to cleave DNA at specific sites. During

repair of these breaks, the cellular repair pathway often introduces small mutations at the break site, which makes it possible to disrupt the ability of the targeted locus to express a functional protein, in this case CCR5. Here, we review the current promise and limitations of CCR5 gene editing with engineered nucleases, including factors affecting the efficiency of gene disruption and potential off-target effects.

Dever, D. P. and M. H. Porteus (2017). "The changing landscape of gene editing in hematopoietic stem cells: a step towards Cas9 clinical translation." Curr Opin Hematol **24**(6): 481-488.

PURPOSE OF REVIEW: Since the discovery two decades ago that programmable endonucleases can be engineered to modify human cells at single nucleotide resolution, the concept of genome editing was born. Now these technologies are being applied to therapeutically relevant cell types, including hematopoietic stem cells (HSC), which possess the power to repopulate an entire blood and immune system. The purpose of this review is to discuss the changing landscape of genome editing in hematopoietic stem cells (GE-HSC) from the discovery stage to the preclinical stage, with the imminent goal of clinical translation for the treatment of serious genetic diseases of the blood and immune system. RECENT FINDINGS: With the discovery that the RNA-programmable (sgRNA) clustered regularly interspace short palindromic repeats (CRISPR)-Cas9 nuclease (Cas9/sgRNA) systems can be easily used to precisely modify the human genome in 2012, a genome-editing revolution of hematopoietic stem cells (HSC) has bloomed. We have observed that over the last 2 years, academic institutions and small biotech companies are developing HSC-based Cas9/sgRNA genome-editing curative strategies to treat monogenic disorders, including beta-hemoglobinopathies and primary immunodeficiencies. We will focus on recent publications (within the past 2 years) that employ different genome-editing strategies to 'hijack' the cell's endogenous double-strand repair pathways to confer a disease-specific therapeutic advantage. SUMMARY: The number of genome-editing strategies in HSCs that could offer therapeutic potential for diseases of the blood and immune system have dramatically risen over the past 2 years. The HSC-based genome-editing field is primed to enter clinical trials in the subsequent years. We will summarize the major advancements for the development of novel autologous GE-HSC cell and gene therapy strategies for hematopoietic diseases that are candidates for curative allogeneic bone marrow transplantation.

Droz-Georget Lathion, S., et al. (2015). "A single epidermal stem cell strategy for safe ex vivo gene therapy." EMBO Mol Med **7**(4): 380-393.

There is a widespread agreement from patient and professional organisations alike that the safety of stem cell therapeutics is of paramount importance, particularly for ex vivo autologous gene therapy. Yet current technology makes it difficult to thoroughly evaluate the behaviour of genetically corrected stem cells before they are transplanted. To address this, we have developed a strategy that permits transplantation of a clonal population of genetically corrected autologous stem cells that meet stringent selection criteria and the principle of precaution. As a proof of concept, we have stably transduced epidermal stem cells (holoclones) obtained from a patient suffering from recessive dystrophic epidermolysis bullosa. Holoclones were infected with self-inactivating retroviruses bearing a COL7A1 cDNA and cloned before the progeny of individual stem cells were characterised using a number of criteria. Clonal analysis revealed a great deal of heterogeneity among transduced stem cells in their capacity to produce functional type VII collagen (COLVII). Selected transduced stem cells transplanted onto immunodeficient mice regenerated a non-blistering epidermis for months and produced a functional COLVII. Safety was assessed by determining the sites of proviral integration, rearrangements and hit genes and by whole-genome sequencing. The progeny of the selected stem cells also had a diploid karyotype, was not tumorigenic and did not disseminate after long-term transplantation onto immunodeficient mice. In conclusion, a clonal strategy is a powerful and efficient means of by-passing the heterogeneity of a transduced stem cell population. It guarantees a safe and homogenous medicinal product, fulfilling the principle of precaution and the requirements of regulatory affairs. Furthermore, a clonal strategy makes it possible to envision exciting gene-editing technologies like zinc finger nucleases, TALENs and homologous recombination for next-generation gene therapy.

Dunbar, C. E., et al. (2018). "Gene therapy comes of age." Science **359**(6372).

After almost 30 years of promise tempered by setbacks, gene therapies are rapidly becoming a critical component of the therapeutic armamentarium for a variety of inherited and acquired human diseases. Gene therapies for inherited immune disorders, hemophilia, eye and neurodegenerative disorders, and lymphoid cancers recently progressed to approved drug status in the United States and Europe, or are anticipated to receive approval in the near future. In this Review, we discuss milestones in the development

of gene therapies, focusing on direct in vivo administration of viral vectors and adoptive transfer of genetically engineered T cells or hematopoietic stem cells. We also discuss emerging genome editing technologies that should further advance the scope and efficacy of gene therapy approaches.

Ewart, D. T., et al. (2018). "Gene editing for inflammatory disorders." Ann Rheum Dis.

Technology for precise and efficient genetic editing is constantly evolving and is now capable of human clinical applications. Autoimmune and inflammatory diseases are chronic, disabling, sometimes life-threatening, conditions that feature heritable components. Both primary genetic lesions and the inflammatory pathobiology underlying these diseases represent fertile soil for new therapies based on the capabilities of gene editing. The ability to orchestrate precise targeted modifications to the genome will likely enable cell-based therapies for inflammatory diseases such as monogenic autoinflammatory disease, acquired autoimmune disease and for regenerative medicine in the setting of an inflammatory environment. Here, we discuss recent advances in genome editing and their evolving applications in immunoinflammatory diseases. Strengths and limitations of older genetic modification tools are compared with CRISPR/Cas9, base editing, RNA editing, targeted activators and repressors of transcription and targeted epigenetic modifiers. Commonly employed delivery vehicles to target cells or tissues of interest with genetic modification machinery, including viral, non-viral and cellular vectors, are described. Finally, applications in animal and human models of inflammatory diseases are discussed. Use of chimeric autoantigen receptor T cells, correction of monogenic diseases with genetically edited haematopoietic stem and progenitor cells, engineering of induced pluripotent stem cells and ex vivo expansion and modification of regulatory T cells for a range of chronic inflammatory diseases are reviewed.

Finotti, A., et al. (2015). "Recent trends in the gene therapy of beta-thalassemia." J Blood Med **6**: 69-85.

The beta-thalassemias are a group of hereditary hematological diseases caused by over 300 mutations of the adult beta-globin gene. Together with sickle cell anemia, thalassemia syndromes are among the most impactful diseases in developing countries, in which the lack of genetic counseling and prenatal diagnosis have contributed to the maintenance of a very high frequency of these genetic diseases in the population. Gene therapy for beta-thalassemia has recently seen steadily accelerating progress and has reached a crossroads in its development. Presently, data from past and ongoing clinical trials guide the design of further clinical and preclinical studies based on gene augmentation, while fundamental insights into globin switching and new technology developments have inspired the investigation of novel gene-therapy approaches. Moreover, human erythropoietic stem cells from beta-thalassemia patients have been the cellular targets of choice to date whereas future gene-therapy studies might increasingly draw on induced pluripotent stem cells. Herein, we summarize the most significant developments in beta-thalassemia gene therapy over the last decade, with a strong emphasis on the most recent findings, for beta-thalassemia model systems; for beta-, gamma-, and anti-sickling beta-globin gene addition and combinatorial approaches including the latest results of clinical trials; and for novel approaches, such as transgene-mediated activation of gamma-globin and genome editing using designer nucleases.

Garate, Z., et al. (2013). "New frontier in regenerative medicine: site-specific gene correction in patient-specific induced pluripotent stem cells." Hum Gene Ther **24**(6): 571-583.

Advances in cell and gene therapy are opening up new avenues for regenerative medicine. Because of their acquired pluripotency, human induced pluripotent stem cells (hiPSCs) are a promising source of autologous cells for regenerative medicine. They show unlimited self-renewal while retaining the ability, in principle, to differentiate into any cell type of the human body. Since Yamanaka and colleagues first reported the generation of hiPSCs in 2007, significant efforts have been made to understand the reprogramming process and to generate hiPSCs with potential for clinical use. On the other hand, the development of gene-editing platforms to increase homologous recombination efficiency, namely DNA nucleases (zinc finger nucleases, TAL effector nucleases, and meganucleases), is making the application of locus-specific gene therapy in human cells an achievable goal. The generation of patient-specific hiPSC, together with gene correction by homologous recombination, will potentially allow for their clinical application in the near future. In fact, reports have shown targeted gene correction through DNA-Nucleases in patient-specific hiPSCs. Various technologies have been described to reprogram patient cells and to correct these patient hiPSCs. However, no approach has been clearly more efficient and safer than the others. In addition, there are still significant challenges for the clinical application of these technologies, such as inefficient differentiation protocols, genetic instability resulting from the reprogramming process and hiPSC culture itself, the

efficacy and specificity of the engineered DNA nucleases, and the overall homologous recombination efficiency. To summarize advances in the generation of gene corrected patient-specific hiPSCs, this review focuses on the available technological platforms, including their strengths and limitations regarding future therapeutic use of gene-corrected hiPSCs.

Goncalves, G. A. R. and R. M. A. Paiva (2017). "Gene therapy: advances, challenges and perspectives." Einstein (Sao Paulo) **15**(3): 369-375.

The ability to make site-specific modifications to the human genome has been an objective in medicine since the recognition of the gene as the basic unit of heredity. Thus, gene therapy is understood as the ability of genetic improvement through the correction of altered (mutated) genes or site-specific modifications that target therapeutic treatment. This therapy became possible through the advances of genetics and bioengineering that enabled manipulating vectors for delivery of extrachromosomal material to target cells. One of the main focuses of this technique is the optimization of delivery vehicles (vectors) that are mostly plasmids, nanostructured or viruses. The viruses are more often investigated due to their excellence of invading cells and inserting their genetic material. However, there is great concern regarding exacerbated immune responses and genome manipulation, especially in germ line cells. In vivo studies in in somatic cell showed satisfactory results with approved protocols in clinical trials. These trials have been conducted in the United States, Europe, Australia and China. Recent biotechnological advances, such as induced pluripotent stem cells in patients with liver diseases, chimeric antigen receptor T-cell immunotherapy, and genomic editing by CRISPR/Cas9, are addressed in this review.

Guo, Q., et al. (2018). "'Cold shock' increases the frequency of homology directed repair gene editing in induced pluripotent stem cells." Sci Rep **8**(1): 2080.

Using CRISPR/Cas9 delivered as a RNA modality in conjunction with a lipid specifically formulated for large RNA molecules, we demonstrate that homology directed repair (HDR) rates between 20-40% can be achieved in induced pluripotent stem cells (iPSC). Furthermore, low HDR rates (between 1-20%) can be enhanced two- to ten-fold in both iPSCs and HEK293 cells by 'cold shocking' cells at 32 degrees C for 24-48 hours following transfection. This method can also increases the proportion of loci that have undergone complete sequence conversion across the donor sequence, or 'perfect HDR', as opposed to partial sequence conversion where nucleotides more distal to the CRISPR cut site are less efficiently incorporated ('partial HDR'). We demonstrate that the structure of the single-stranded DNA oligo donor can influence the fidelity of HDR, with oligos symmetric with respect to the CRISPR cleavage site and complementary to the target strand being more efficient at directing 'perfect HDR' compared to asymmetric non-target strand complementary oligos. Our protocol represents an efficient method for making CRISPR-mediated, specific DNA sequence changes within the genome that will facilitate the rapid generation of genetic models of human disease in iPSCs as well as other genome engineered cell lines.

Gupta, R. M., et al. (2017). "A Genetic Variant Associated with Five Vascular Diseases Is a Distal Regulator of Endothelin-1 Gene Expression." Cell **170**(3): 522-533 e515.

Genome-wide association studies (GWASs) implicate the PHACTR1 locus (6p24) in risk for five vascular diseases, including coronary artery disease, migraine headache, cervical artery dissection, fibromuscular dysplasia, and hypertension. Through genetic fine mapping, we prioritized rs9349379, a common SNP in the third intron of the PHACTR1 gene, as the putative causal variant. Epigenomic data from human tissue revealed an enhancer signature at rs9349379 exclusively in aorta, suggesting a regulatory function for this SNP in the vasculature. CRISPR-edited stem cell-derived endothelial cells demonstrate rs9349379 regulates expression of endothelin 1 (EDN1), a gene located 600 kb upstream of PHACTR1. The known physiologic effects of EDN1 on the vasculature may explain the pattern of risk for the five associated diseases. Overall, these data illustrate the integration of genetic, phenotypic, and epigenetic analysis to identify the biologic mechanism by which a common, non-coding variant can distally regulate a gene and contribute to the pathogenesis of multiple vascular diseases.

Heman-Ackah, S. M., et al. (2016). "Precision Modulation of Neurodegenerative Disease-Related Gene Expression in Human iPSC-Derived Neurons." Sci Rep **6**: 28420.

The ability to reprogram adult somatic cells into induced pluripotent stem cells (iPSCs) and the subsequent development of protocols for their differentiation into disease-relevant cell types have enabled in-depth molecular analyses of multiple disease states as hitherto impossible. Neurons differentiated from patient-specific iPSCs provide a means to recapitulate molecular phenotypes of neurodegenerative diseases in vitro. However, it remains challenging to conduct precise manipulations of gene expression in iPSC-derived neurons towards modeling complex human neurological diseases. The application of CRISPR/Cas9 to mammalian systems is

revolutionizing the utilization of genome editing technologies in the study of molecular contributors to the pathogenesis of numerous diseases. Here, we demonstrate that CRISPRa and CRISPRi can be used to exert precise modulations of endogenous gene expression in fate-committed iPSC-derived neurons. This highlights CRISPRa/i as a major technical advancement in accessible tools for evaluating the specific contributions of critical neurodegenerative disease-related genes to neuropathogenesis.

Hendriks, W. T., et al. (2015). "TALEN- and CRISPR/Cas9-Mediated Gene Editing in Human Pluripotent Stem Cells Using Lipid-Based Transfection." Curr Protoc Stem Cell Biol **34**: 5B 3 1-25.

Using custom-engineered nuclease-mediated genome editing, such as Transcription Activator-Like Effector Nucleases (TALENs) and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) RNA-guided Cas9 nucleases, human pluripotent stem cell (hPSC) lines with knockout or mutant alleles can be generated and differentiated into various cell types. This strategy of genome engineering in hPSCs will prove invaluable for studying human biology and disease. Here, we provide a detailed protocol for design and construction of TALEN and CRISPR vectors, testing of their nuclease activity, and delivery of TALEN or CRISPR vectors into hPSCs. In addition, we describe the use of single-stranded oligodeoxynucleotides (ssODNs) to introduce or repair point mutations. Next, we describe the identification of edited hPSC clones without antibiotic selection, including their clonal selection, genotyping, and expansion for downstream applications.

Hiramoto, T., et al. (2018). "Nuclease-free Adeno-Associated Virus-Mediated Il2rg Gene Editing in X-SCID Mice." Mol Ther **26**(5): 1255-1265.

X-linked severe combined immunodeficiency (X-SCID) has been successfully treated by hematopoietic stem cell (HSC) transduction with retroviral vectors expressing the interleukin-2 receptor subunit gamma gene (IL2RG), but several patients developed malignancies due to vector integration near cellular oncogenes. This adverse side effect could in principle be avoided by accurate IL2RG gene editing with a vector that does not contain a functional promoter or IL2RG gene. Here, we show that adeno-associated virus (AAV) gene editing vectors can insert a partial Il2rg cDNA at the endogenous Il2rg locus in X-SCID murine bone marrow cells and that these ex vivo-edited cells repopulate transplant recipients and produce CD4(+) and CD8(+) T cells. Circulating, edited lymphocytes increased over time and appeared in secondary transplant recipients, demonstrating successful editing in long-term repopulating cells. Random vector integration events were nearly undetectable, and malignant transformation of the transplanted cells was not observed. Similar editing frequencies were observed in human hematopoietic cells. Our results demonstrate that therapeutically relevant HSC gene editing can be achieved by AAV vectors in the absence of site-specific nucleases and suggest that this may be a safe and effective therapy for hematopoietic diseases where in vivo selection can increase edited cell numbers.

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. 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.

Howden, S. E., et al. (2018). "Simultaneous reprogramming and gene editing of human fibroblasts." Nat Protoc **13**(5): 875-898.

The utility of human induced pluripotent stem cells (iPSCs) is enhanced by an ability to precisely modify a chosen locus with minimal impact on the remaining genome. However, the derivation of gene-edited iPSCs typically involves multiple steps requiring lengthy culture periods and several clonal events. Here, we describe a one-step protocol for reliable generation of clonally derived gene-edited iPSC lines from human fibroblasts in the absence of drug selection or FACS enrichment. Using enhanced episomal-based reprogramming and CRISPR/Cas9 systems, gene-edited and passage-matched unmodified iPSC lines are obtained following a single electroporation of human fibroblasts. To minimize unwanted mutations within the target locus, we use a Cas9 variant that is associated with decreased nonhomologous end-joining (NHEJ) activity. This protocol outlines in detail how this streamlined approach can be used for both monoallelic and biallelic introduction of specific base changes or transgene cassettes in a manner that is efficient, rapid (approximately 6-8 weeks), and cost-effective.

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.

Jang, Y. Y. and Z. Ye (2016). "Gene correction in patient-specific iPSCs for therapy development and disease modeling." Hum Genet **135**(9): 1041-1058.

The discovery that mature cells can be reprogrammed to become pluripotent and the development of engineered endonucleases for enhancing genome editing are two of the most exciting and impactful technology advances in modern medicine and science. Human pluripotent stem cells have the potential to establish new model systems for studying human developmental biology and disease mechanisms. Gene correction in patient-specific iPSCs can also provide a novel source for autologous cell therapy. Although historically challenging, precise genome editing in human iPSCs is becoming more feasible with the development of new genome-editing tools, including ZFNs, TALENs, and CRISPR. iPSCs derived from patients of a variety of diseases have been edited to correct disease-associated mutations and to generate isogenic cell lines. After directed differentiation, many of the corrected iPSCs showed restored functionality and demonstrated their potential in cell replacement therapy. Genome-wide analyses of gene-corrected iPSCs have collectively demonstrated a high fidelity of the engineered endonucleases. Remaining challenges in clinical translation of these technologies include maintaining genome integrity of the iPSC clones and the differentiated cells. Given the rapid advances in genome-editing technologies, gene correction is no longer the bottleneck in developing iPSC-based gene and cell therapies; generating functional and transplantable cell types from iPSCs remains the biggest challenge needing to be addressed by the research field.

Jeong, I. S., et al. (2018). "Dual chemotactic factors-secreting human amniotic mesenchymal stem cells via TALEN-mediated gene editing enhanced angiogenesis." Int J Cardiol **260**: 156-162.

BACKGROUND: Even though mesenchymal stem cells (MSCs) have angiogenic property, their cytokine secretory capacity is limited to treat ischemic vascular disorders. In present study, we produced genome-edited MSCs that secreted dual chemokine granulocyte chemotactic protein-2 (GCP-2) and stromal-derived factor-1alpha (SDF-1alpha) and determined their therapeutic potential in the context of experimental ischemia. METHODS: GCP-2 and SDF-1alpha genes were integrated into safe harbor site at the safe harbor genomic locus of amniotic mesenchymal stem cells (AMM) via transcription activator-like effector nucleases (TALEN). GCP-2 and SDF-1alpha gene-edited AMM (AMM/GS) were used for quantitative (q)-PCR, Matrigel tube formation, cell migration, Matrigel plug assays and in vivo therapeutic assays using hindlimb ischemia mouse model. RESULTS: AMM/GS-derived culture media (CM) induced significantly higher tube lengths and branching points as compared to AMM/S CM and AMM CM. Interestingly, Matrigel plug assays revealed that significantly higher levels of red blood cells were found in AMM/GS than AMM/S and AMM Matigel plugs and exhibited micro-vascular like formation. Cells was transplanted into ischemic mouse hindlimbs and compared with control groups. AMM/GS injection prevented limb loss and augmented blood perfusion, suggesting that enhances neovascularization in hindlimb ischemia. In addition, transplanted AMM/GS revealed high vasculogenic potential in vivo compared with transplanted AMM/S. CONCLUSION: Taken together, genome-edited MSCs that express dual chemokine GCP-2 and SDF-1alpha might be alternative therapeutic options for the treatment of ischemic vascular disease.

Jia, J., et al. (2015). "Efficient Gene Editing in Pluripotent Stem Cells by Bacterial Injection of Transcription Activator-Like Effector Nuclease Proteins." Stem Cells Transl Med **4**(8): 913-926.

The type III secretion system (T3SS) of Pseudomonas aeruginosa is a powerful tool for direct protein delivery into mammalian cells and has successfully been used to deliver various exogenous proteins into mammalian cells. In the present study, transcription activator-like effector nuclease (TALEN) proteins have been efficiently delivered using the P. aeruginosa T3SS into mouse embryonic stem cells (mESCs), human ESCs (hESCs), and human induced pluripotent stem cells (hiPSCs) for genome editing. This bacterial delivery system offers an alternative method of TALEN delivery that is highly efficient in cleavage of the chromosomal target and presumably safer by avoiding plasmid DNA introduction. We combined the method of bacterial T3SS-mediated TALEN protein injection and transfection of an oligonucleotide template to effectively generate precise genetic modifications in the stem cells. Initially, we efficiently edited a single-base in the gfp gene of a mESC line to silence green fluorescent protein (GFP) production. The resulting GFP-negative mESC was cloned from a single cell and subsequently mutated back to a GFP-positive mESC line. Using the same approach, the gfp gene was also effectively knocked out in hESCs. In addition, a defined single-base edition was effectively introduced into the X-chromosome-linked HPRT1 gene in hiPSCs, generating an in vitro model of Lesch-Nyhan syndrome. T3SS-mediated TALEN protein delivery provides a highly efficient alternative for introducing precise gene editing within pluripotent stem cells for the purpose of disease genotype-phenotype relationship studies and cellular replacement therapies.

Jiang, D. J., et al. (2018). "Revolution in Gene Medicine Therapy and Genome Surgery." Genes (Basel) **9**(12).

Recently, there have been revolutions in the development of both gene medicine therapy and genome surgical treatments for inherited disorders. Much of this progress has been centered on hereditary retinal dystrophies, because the eye is an immune-privileged and anatomically ideal target. Gene therapy treatments, already demonstrated to be safe and efficacious in numerous clinical trials, are benefitting from the development of new viral vectors, such as dual and triple adeno-associated virus (AAV) vectors. CRISPR/Cas9, which revolutionized the field of gene editing, is being adapted into more precise "high fidelity" and catalytically dead variants. Newer CRISPR endonucleases, such as CjCas9 and Cas12a, are generating excitement in the field as well. Stem cell therapy has emerged as a promising alternative, allowing human embryo-derived stem cells and induced pluripotent stem cells to be edited precisely in vitro and then reintroduced into the body. This article highlights recent progress made in gene therapy and genome surgery for retinal disorders, and it provides an update on precision medicine Food and Drug Administration (FDA) treatment trials.

Kehler, J., et al. (2017). "RNA-Generated and Gene-Edited Induced Pluripotent Stem Cells for Disease Modeling and Therapy." J Cell Physiol **232**(6): 1262-1269.

Cellular reprogramming by epigenomic remodeling of chromatin holds great promise in the field of human regenerative medicine. As an example,

human-induced Pluripotent Stem Cells (iPSCs) obtained by reprograming of patient somatic cells are sufficiently similar to embryonic stem cells (ESCs) and can generate all cell types of the human body. Clinical use of iPSCs is dependent on methods that do not utilize genome altering transgenic technologies that are potentially unsafe and ethically unacceptable. Transient delivery of exogenous RNA into cells provides a safer reprogramming system to transgenic approaches that rely on exogenous DNA or viral vectors. RNA reprogramming may prove to be more suitable for clinical applications and provide stable starting cell lines for gene-editing, isolation, and characterization of patient iPSC lines. The introduction and rapid evolution of CRISPR/Cas9 gene-editing systems has provided a readily accessible research tool to perform functional human genetic experiments. Similar to RNA reprogramming, transient delivery of mRNA encoding Cas9 in combination with guide RNA sequences to target specific points in the genome eliminates the risk of potential integration of Cas9 plasmid constructs. We present optimized RNA-based laboratory procedure for making and editing iPSCs. In the near-term these two powerful technologies are being harnessed to dissect mechanisms of human development and disease in vitro, supporting both basic, and translational research. J. Cell. Physiol. 232: 1262-1269, 2017. (c) 2016 Wiley Periodicals, Inc.

Kim, E. J., et al. (2017). "CRISPR-Cas9: a promising tool for gene editing on induced pluripotent stem cells." Korean J Intern Med **32**(1): 42-61.

Recent advances in genome editing with programmable nucleases have opened up new avenues for multiple applications, from basic research to clinical therapy. The ease of use of the technology-and particularly clustered regularly interspaced short palindromic repeats (CRISPR)-will allow us to improve our understanding of genomic variation in disease processes via cellular and animal models. Here, we highlight the progress made in correcting gene mutations in monogenic hereditary disorders and discuss various CRISPR-associated applications, such as cancer research, synthetic biology, and gene therapy using induced pluripotent stem cells. The challenges, ethical issues, and future prospects of CRISPR-based systems for human research are also discussed.

Kim, S. J., et al. (2017). "A homozygous Keap1-knockout human embryonic stem cell line generated using CRISPR/Cas9 mediates gene targeting." Stem Cell Res **19**: 52-54.

Kelch-like ECH-associated protein 1 (keap1) is a cysteine-rich protein that interacts with transcription factor Nrf2 in a redox-sensitive manner, leading to the degradation of Nrf2 (Kim et al., 2014a). Disruption of Keap1 results in the induction of Nrf2-related signaling pathways involving the expression of a set of anti-oxidant and anti-inflammatory genes. We generated biallelic mutants of the Keap1 gene using a CRISPR-Cas9 genome editing method in the H9 human embryonic stem cell (hESC). The Keap1 homozygous-knockout H9 cell line retained normal morphology, gene expression, and in vivo differentiation potential.

Kim, Y. K., et al. (2017). "Gene-Edited Human Kidney Organoids Reveal Mechanisms of Disease in Podocyte Development." Stem Cells **35**(12): 2366-2378.

A critical event during kidney organogenesis is the differentiation of podocytes, specialized epithelial cells that filter blood plasma to form urine. Podocytes derived from human pluripotent stem cells (hPSC-podocytes) have recently been generated in nephron-like kidney organoids, but the developmental stage of these cells and their capacity to reveal disease mechanisms remains unclear. Here, we show that hPSC-podocytes phenocopy mammalian podocytes at the capillary loop stage (CLS), recapitulating key features of ultrastructure, gene expression, and mutant phenotype. hPSC-podocytes in vitro progressively establish junction-rich basal membranes (nephrin (+) podocin (+) ZO-1(+)) and microvillus-rich apical membranes (podocalyxin (+)), similar to CLS podocytes in vivo. Ultrastructural, biophysical, and transcriptomic analysis of podocalyxin-knockout hPSCs and derived podocytes, generated using CRISPR/Cas9, reveals defects in the assembly of microvilli and lateral spaces between developing podocytes, resulting in failed junctional migration. These defects are phenocopied in CLS glomeruli of podocalyxin-deficient mice, which cannot produce urine, thereby demonstrating that podocalyxin has a conserved and essential role in mammalian podocyte maturation. Defining the maturity of hPSC-podocytes and their capacity to reveal and recapitulate pathophysiological mechanisms establishes a powerful framework for studying human kidney disease and regeneration. Stem Cells 2017;35:2366-2378.

Laskowski, T. J., et al. (2016). "Gene Correction of iPSCs from a Wiskott-Aldrich Syndrome Patient Normalizes the Lymphoid Developmental and Functional Defects." Stem Cell Reports **7**(2): 139-148.

Wiskott-Aldrich syndrome (WAS) is an X-linked primary immunodeficiency disease caused by mutations in the gene encoding the WAS protein (WASp). Here, induced pluripotent stem cells (iPSCs) were derived from a WAS patient (WAS-iPSC) and the endogenous chromosomal WAS locus was targeted with a wtWAS-2A-eGFP transgene using zinc finger

nucleases (ZFNs) to generate corrected WAS-iPSC (cWAS-iPSC). WASp and GFP were first expressed in the earliest CD34(+)CD43(+)CD45(-) hematopoietic precursor cells and later in all hematopoietic lineages examined. Whereas differentiation to non-lymphoid lineages was readily obtained from WAS-iPSCs, in vitro T lymphopoiesis from WAS-iPSC was deficient with few CD4(+)CD8(+) double-positive and mature CD3(+) T cells obtained. T cell differentiation was restored for cWAS-iPSCs. Similarly, defects in natural killer cell differentiation and function were restored on targeted correction of the WAS locus. These results demonstrate that the defects exhibited by WAS-iPSC-derived lymphoid cells were fully corrected and suggests the potential therapeutic use of gene-corrected WAS-iPSCs.

Lau, R. W., et al. (2018). "Gene editing of stem cells for kidney disease modelling and therapeutic intervention." Nephrology (Carlton) **23**(11): 981-990.

Recent developments in targeted gene editing have paved the way for the wide adoption of clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein-9 nucleases (Cas9) as an RNA-guided molecular tool to modify the genome of eukaryotic cells of animals. Theoretically, the translation of CRISPR-Cas9 can be applied to the treatment of inherited or acquired kidney disease, kidney transplantation and genetic corrections of somatic cells from kidneys with inherited mutations, such as polycystic kidney disease. Human pluripotent stem cells have been used to generate an unlimited source of kidney progenitor cells or, when spontaneously differentiated into three-dimensional kidney organoids, to model kidney organogenesis or the pathogenesis of disease. Gene editing now allows for the tagging and selection of specific kidney cell types or disease-specific gene knock in/out, which enables more precise understanding of kidney organogenesis and genetic diseases. This review discusses the mechanisms of action, in addition to the advantages and disadvantages, of the three major gene editing technologies, namely, CRISPR-Cas9, zinc finger nucleases and transcription activator-like effector nucleases. The implications of using gene editing to better understand kidney disease is reviewed in detail. In addition, the ethical issues of gene editing, which could be easily neglected in the modern, fast-paced research environment, are highlighted.

Lee, C. M., et al. (2017). "Design and Validation of CRISPR/Cas9 Systems for Targeted Gene Modification in Induced Pluripotent Stem Cells." Methods Mol Biol **1498**: 3-21.

The CRISPR/Cas9 system is a powerful tool for precision genome editing. The ability to accurately modify genomic DNA in situ with single nucleotide precision opens up new possibilities for not only basic research but also biotechnology applications and clinical translation. In this chapter, we outline the procedures for design, screening, and validation of CRISPR/Cas9 systems for targeted modification of coding sequences in the human genome and how to perform genome editing in induced pluripotent stem cells with high efficiency and specificity.

Lei, Y., et al. (2011). "Gene editing of human embryonic stem cells via an engineered baculoviral vector carrying zinc-finger nucleases." Mol Ther **19**(5): 942-950.

Human embryonic stem (hES) cells are renewable cell sources that have potential applications in regenerative medicine. The development of technologies to produce permanent and site-specific genome modifications is in demand to achieve future medical implementation of hES cells. We report herein that a baculoviral vector (BV) system carrying zinc-finger nucleases (ZFNs) can successfully modify the hES cell genome. BV-mediated transient expression of ZFNs specifically disrupted the CCR5 locus in transduced cells and the modified cells exhibited resistance to HIV-1 transduction. To convert the BV to a gene targeting vector, a DNA donor template and ZFNs were incorporated into the vector. These hybrid vectors yielded permanent site-specific gene addition in both immortalized human cell lines (10%) and hES cells (5%). Modified hES cells were both karyotypically normal and pluripotent. These results suggest that this baculoviral delivery system can be engineered for site-specific genetic manipulation in hES cells.

Li, M., et al. (2017). "Establishment of Reporter Lines for Detecting Fragile X Mental Retardation (FMR1) Gene Reactivation in Human Neural Cells." Stem Cells **35**(1): 158-169.

Human patient-derived induced pluripotent stem cells (hiPSCs) provide unique opportunities for disease modeling and drug development. However, adapting hiPSCs or their differentiated progenies to high throughput assays for phenotyping or drug screening has been challenging. Fragile X syndrome (FXS) is the most common inherited cause of intellectual disability and a major genetic cause of autism. FXS is caused by mutational trinucleotide expansion in the FMR1 gene leading to hypermethylation and gene silencing. One potential therapeutic strategy is to reactivate the silenced FMR1 gene, which has been attempted using both candidate chemicals and cell-based screening. However, molecules that effectively reactivate the silenced FMR1 gene are yet to be identified; therefore, a high throughput unbiased screen is needed. Here we

demonstrate the creation of a robust FMR1-Nluc reporter hiPSC line by knocking in a Nano luciferase (Nluc) gene into the endogenous human FMR1 gene using the CRISPR/Cas9 genome editing method. We confirmed that luciferase activities faithfully report FMR1 gene expression levels and showed that neural progenitor cells derived from this line could be optimized for high throughput screening. The FMR1-Nluc reporter line is a good resource for drug screening as well as for testing potential genetic reactivation strategies. In addition, our data provide valuable information for the generation of knockin human iPSC reporter lines for disease modeling, drug screening, and mechanistic studies. Stem Cells 2017;35:158-169.

Liao, H. K., et al. (2017). "In Vivo Target Gene Activation via CRISPR/Cas9-Mediated Trans-epigenetic Modulation." Cell **171**(7): 1495-1507 e1415.

Current genome-editing systems generally rely on inducing DNA double-strand breaks (DSBs). This may limit their utility in clinical therapies, as unwanted mutations caused by DSBs can have deleterious effects. CRISPR/Cas9 system has recently been repurposed to enable target gene activation, allowing regulation of endogenous gene expression without creating DSBs. However, in vivo implementation of this gain-of-function system has proven difficult. Here, we report a robust system for in vivo activation of endogenous target genes through trans-epigenetic remodeling. The system relies on recruitment of Cas9 and transcriptional activation complexes to target loci by modified single guide RNAs. As proof-of-concept, we used this technology to treat mouse models of diabetes, muscular dystrophy, and acute kidney disease. Results demonstrate that CRISPR/Cas9-mediated target gene activation can be achieved in vivo, leading to measurable phenotypes and amelioration of disease symptoms. This establishes new avenues for developing targeted epigenetic therapies against human diseases. VIDEO ABSTRACT.

Lidonnici, M. R. and G. Ferrari (2018). "Gene therapy and gene editing strategies for hemoglobinopathies." Blood Cells Mol Dis **70**: 87-101.

Gene therapy for hemoglobinopathies is currently based on transplantation of autologous hematopoietic stem cells genetically modified with an integrating lentiviral vector expressing a globin gene under the control of globin transcriptional regulatory elements. Studies and safety works demonstrated the potential therapeutic efficacy and safety of this approach, providing the rationale for clinical translation. The outcomes of early clinical trials, although showing promising results, have highlighted the current limitations to a more general application. These include the nature, source and age of repopulating hematopoietic stem cells, the suboptimal transduction efficiency and gene expression levels, the toxicity and efficacy of bone marrow conditioning, the stress status of bone marrow microenvironment in chronic diseases such as beta-thalassemia and sickle cell disease. Recently, gene editing strategies based on the use of nucleases offered a novel approach to increase globin expression in a quasi-physiological way, independently from the addition of transgenes and viral sequences to the human genome. This review will discuss the current status of gene therapy for beta-thalassemia and sickle cell disease with a perspective towards the improvements necessary in the context of clinical translation.

Lombardo, A., et al. (2007). "Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery." Nat Biotechnol **25**(11): 1298-1306.

Achieving the full potential of zinc-finger nucleases (ZFNs) for genome engineering in human cells requires their efficient delivery to the relevant cell types. Here we exploited the infectivity of integrase-defective lentiviral vectors (IDLV) to express ZFNs and provide the template DNA for gene correction in different cell types. IDLV-mediated delivery supported high rates (13-39%) of editing at the IL-2 receptor common gamma-chain gene (IL2RG) across different cell types. IDLVs also mediated site-specific gene addition by a process that required ZFN cleavage and homologous template DNA, thus establishing a platform that can target the insertion of transgenes into a predetermined genomic site. Using IDLV delivery and ZFNs targeting distinct loci, we observed high levels of gene addition (up to 50%) in a panel of human cell lines, as well as human embryonic stem cells (5%), allowing rapid, selection-free isolation of clonogenic cells with the desired genetic modification.

Marotta, P., et al. (2018). "Combining cell and gene therapy to advance cardiac regeneration." Expert Opin Biol Ther **18**(4): 409-423.

INTRODUCTION: The characterization of multipotent endogenous cardiac stem cells (eCSCs) and the breakthroughs of somatic cell reprogramming to boost cardiomyocyte replacement have fostered the prospect of achieving functional heart repair/regeneration. Areas covered: Allogeneic CSC therapy through its paracrine stimulation of the endogenous resident reparative/regenerative process produces functional meaningful myocardial regeneration in pre-clinical porcine myocardial infarction models and is currently tested in the first-in-

man human trial. The in vivo test of somatic reprogramming and cardioregenerative non-coding RNAs revived the interest in gene therapy for myocardial regeneration. The latter, together with the advent of genome editing, has prompted most recent efforts to produce genetically-modified allogeneic CSCs that secrete cardioregenerative factors to optimize effective myocardial repair. Expert opinion: The current war against heart failure epidemics in western countries seeks to find effective treatments to set back the failing hearts prolonging human lifespan. Off-the-shelf allogeneic-genetically-modified CSCs producing regenerative agents are a novel and evolving therapy set to be affordable, safe, effective and available at all times for myocardial regeneration to either prevent or treat heart failure.

Matsubara, Y., et al. (2014). "Transcription activator-like effector nuclease-mediated transduction of exogenous gene into IL2RG locus." Sci Rep **4**: 5043.

X-linked severe combined immunodeficiency (SCID-X1) caused by mutations in interleukin 2 receptor gamma (IL2RG) gene threatens the survival of affected boys during the first year of life unless hematopoietic stem cell transplantation is provided. Although viral vector-mediated gene therapy has been successfully performed in patients with no HLA-matched donors, leukemia caused by vector-mediated insertional mutagenesis has been reported in some individuals. Transcription activator-like effector nuclease (TALEN) is an artificial sequence-specific endonuclease that is expected to revolutionize the precise correction of disease-causing mutations and eliminate the risk of insertional mutagenesis. Here, we report TALEN-mediated genome editing of the IL2RG locus. We transfected TALENs along with a targeting vector into Jurkat cells, and we confirmed the precise introduction of the exogenous gene into the IL2RG locus. In addition, we found that the length of homology arm in the targeting vector influenced the efficiency of TALEN-mediated homologous recombination.

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.

Merkert, S. and U. Martin (2018). "Targeted Gene Editing in Human Pluripotent Stem Cells Using Site-Specific Nucleases." Adv Biochem Eng Biotechnol **163**: 169-186.

Introduction of induced pluripotent stem cell (iPSC) technology and site-directed nucleases brought a major breakthrough in the development of regenerative therapies and biomedical research. With the advancement of ZFNs, TALENs, and the CRISPR/Cas9 technology, straightforward and precise manipulation of the genome of human pluripotent stem cells (PSC) became possible, allowing relatively easy and fast generation of gene knockouts, integration of transgenes, or even introduction of single nucleotide changes for correction or introduction of disease-specific mutations. We review current applications of site-specific nucleases in human PSCs and focus on trends and challenges for efficient gene editing and improvement of targeting strategies. Graphical Abstract.

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.

Navarro, S., et al. (2017). "Induced Pluripotency and Gene Editing in Fanconi Anemia." Curr Gene Ther **16**(5): 321-328.

Induced pluripotent stem cells (iPSCs) represent an invaluable tool in a chromosomal instability syndrome such as Fanconi anemia (FA), as they can allow to study of the molecular defects underlying this disease. Many other applications, such as its use as a platform to test different methods or compounds, could also be of interest. But the greatest impact of iPSCs may be in bone marrow failure diseases, as iPSCs could represent an unlimited source of autologous cells to apply in advanced treatments such as gene therapy. At the same time, genome editing constitutes the next generation of technology to further develop a safer, personalized, targeted gene therapy. Despite the promising advantages that these two technologies would present in a disease such as FA, the specific characteristics of the disease make both of these processes especially challenging. Efficient and safer FA-hiPSC (human induced pluripotent stem cell) generation methods, robust and reliable differentiation protocols for iPSCs, as well as really efficient delivery methods to perform targeted gene correction should be developed.

Osborn, M. J., et al. (2013). "TALEN-based gene correction for epidermolysis bullosa." Mol Ther **21**(6): 1151-1159.

Recessive dystrophic epidermolysis bullosa (RDEB) is characterized by a functional deficit of type VII collagen protein due to gene defects in the type VII collagen gene (COL7A1). Gene augmentation therapies are promising, but run the risk of insertional mutagenesis. To abrogate this risk, we explored the possibility of using engineered transcription activator-like effector nucleases (TALEN) for precise genome editing. We report the ability of TALEN to induce site-specific double-stranded DNA breaks (DSBs) leading to homology-directed repair (HDR) from an exogenous donor template. This process resulted in COL7A1 gene mutation correction in primary fibroblasts that were subsequently reprogrammed into inducible pluripotent stem cells and showed normal protein expression and deposition in a teratoma-based skin model in vivo. Deep sequencing-based genome-wide screening established a safety profile showing on-target activity and three off-target (OT) loci that, importantly, were at least 10 kb from a coding sequence. This study provides proof-of-concept for TALEN-mediated in situ correction of an endogenous patient-specific gene mutation and used an unbiased screen for comprehensive TALEN target mapping that will cooperatively facilitate translational application.

Ou, Z., et al. (2016). "The Combination of CRISPR/Cas9 and iPSC Technologies in the Gene Therapy of Human beta-thalassemia in Mice." Sci Rep **6**: 32463.

beta-thalassemia results from point mutations or small deletions in the beta-globin (HBB) gene that ultimately cause anemia. The generation of induced pluripotent stem cells (iPSCs) from the somatic cells of patients in combination with subsequent homologous recombination-based gene correction provides new approaches to cure this disease. CRISPR/Cas9 is a genome editing tool that is creating a buzz in the scientific community for treating human diseases, especially genetic disorders. Here, we reported that correction of beta-thalassemia mutations in patient-specific iPSCs using the CRISPR/Cas9 tool promotes hematopoietic differentiation in vivo. CRISPR/Cas9-corrected iPSC-derived hematopoietic stem cells (HSCs) were injected into sublethally-irradiated NOD-scid-IL2Rg-/- (NSI) mice. HBB expression was observed in these HSCs after hematopoietic differentiation in the NSI mice. Importantly, no tumor was found in the livers, lungs, kidneys, or bone marrow at 10 weeks in the NSI mice after implantation with these HSCs. Collectively, our findings demonstrated that CRISPR/Cas9 successfully corrects beta-thalassemia mutations in patient-specific iPSCs. These CRISPR/Cas9-corrected iPSC-derived HSCs express normal HBB in mice without tumorigenic potential, suggesting a safe strategy for personalized treatment of beta-thalassemia.

Ovando-Roche, P., et al. (2017). "Harnessing the Potential of Human Pluripotent Stem Cells and Gene Editing for the Treatment of Retinal Degeneration." Curr Stem Cell Rep **3**(2): 112-123.

PURPOSE OF REVIEW: A major cause of visual disorders is dysfunction and/or loss of the light-sensitive cells of the retina, the photoreceptors. To develop better treatments for patients, we need to understand how inherited retinal disease mutations result in the dysfunction of photoreceptors. New advances in the field of stem cell and gene editing research offer novel ways to model retinal dystrophies in vitro and present opportunities to translate basic

biological insights into therapies. This brief review will discuss some of the issues that should be taken into account when carrying out disease modelling and gene editing of retinal cells. We will discuss (i) the use of human induced pluripotent stem cells (iPSCs) for disease modelling and cell therapy; (ii) the importance of using isogenic iPSC lines as controls; (iii) CRISPR/Cas9 gene editing of iPSCs; and (iv) in vivo gene editing using AAV vectors. RECENT FINDINGS: Ground-breaking advances in differentiation of iPSCs into retinal organoids and methods to derive mature light sensitive photoreceptors from iPSCs. Furthermore, single AAV systems for in vivo gene editing have been developed which makes retinal in vivo gene editing therapy a real prospect. SUMMARY: Genome editing is becoming a valuable tool for disease modelling and in vivo gene editing in the retina.

Park, A., et al. (2016). "Sendai virus, an RNA virus with no risk of genomic integration, delivers CRISPR/Cas9 for efficient gene editing." Mol Ther Methods Clin Dev **3**: 16057.

The advent of RNA-guided endonuclease (RGEN)-mediated gene editing, specifically via CRISPR/Cas9, has spurred intensive efforts to improve the efficiency of both RGEN delivery and targeted mutagenesis. The major viral vectors in use for delivery of Cas9 and its associated guide RNA, lentiviral and adeno-associated viral systems, have the potential for undesired random integration into the host genome. Here, we repurpose Sendai virus, an RNA virus with no viral DNA phase and that replicates solely in the cytoplasm, as a delivery system for efficient Cas9-mediated gene editing. The high efficiency of Sendai virus infection resulted in high rates of on-target mutagenesis in cell lines (75-98% at various endogenous and transgenic loci) and primary human monocytes (88% at the ccr5 locus) in the absence of any selection. In conjunction with extensive former work on Sendai virus as a promising gene therapy vector that can infect a wide range of cell types including hematopoietic stem cells, this proof-of-concept study opens the door to using Sendai virus as well as other related paramyxoviruses as versatile and efficient tools for gene editing.

Park, C. Y., et al. (2014). "Targeted inversion and reversion of the blood coagulation factor 8 gene in human iPS cells using TALENs." Proc Natl Acad Sci U S A **111**(25): 9253-9258.

Hemophilia A, one of the most common genetic bleeding disorders, is caused by various mutations in the blood coagulation factor VIII (F8) gene. Among the genotypes that result in hemophilia A, two different types of chromosomal inversions that involve a portion of the F8 gene are most frequent, accounting for almost half of all severe hemophilia A cases. In this study, we used a transcription activator-like effector nuclease (TALEN) pair to invert a 140-kbp chromosomal segment that spans the portion of the F8 gene in human induced pluripotent stem cells (iPSCs) to create a hemophilia A model cell line. In addition, we reverted the inverted segment back to its normal orientation in the hemophilia model iPSCs using the same TALEN pair. Importantly, we detected the F8 mRNA in cells derived from the reverted iPSCs lines, but not in those derived from the clones with the inverted segment. Thus, we showed that TALENs can be used both for creating disease models associated with chromosomal rearrangements in iPSCs and for correcting genetic defects caused by chromosomal inversions. This strategy provides an iPSC-based novel therapeutic option for the treatment of hemophilia A and other genetic diseases caused by chromosomal inversions.

Park, K. E., et al. (2017). "Generation of germline ablated male pigs by CRISPR/Cas9 editing of the NANOS2 gene." Sci Rep **7**: 40176.

Genome editing tools have revolutionized the generation of genetically modified animals including livestock. In particular, the domestic pig is a proven model of human physiology and an agriculturally important species. In this study, we utilized the CRISPR/Cas9 system to edit the NANOS2 gene in pig embryos to generate offspring with mono-allelic and bi-allelic mutations. We found that NANOS2 knockout pigs phenocopy knockout mice with male specific germline ablation but other aspects of testicular development are normal. Moreover, male pigs with one intact NANOS2 allele and female knockout pigs are fertile. From an agriculture perspective, NANOS2 knockout male pigs are expected to serve as an ideal surrogate for transplantation of donor spermatogonial stem cells to expand the availability of gametes from genetically desirable sires.

Pu, J., et al. (2015). "Utilization of TALEN and CRISPR/Cas9 technologies for gene targeting and modification." Exp Biol Med (Maywood) **240**(8): 1065-1070.

The capability to modify the genome precisely and efficiently offers an extremely useful tool for biomedical research. Recent developments in genome editing technologies such as transcription activator-like effector nuclease and the clustered regularly interspaced short palindromic repeats system have made genome modification available for a number of organisms with relative ease. Here, we introduce these genome editing techniques, compare and contrast each

technical approach and discuss their potential to study the underlying mechanisms of human disease using patient-derived induced pluripotent stem cells.

Radecke, S., et al. (2018). "Genome-wide Mapping of Off-Target Events in Single-Stranded Oligodeoxynucleotide-Mediated Gene Repair Experiments." Mol Ther **26**(1): 115-131.

Short single-stranded oligodeoxynucleotides are versatile molecular tools used in different applications. They enable gene repair and genome editing, and they are central to the antisense technology. Because the usability of single-stranded oligodeoxynucleotides depends on their efficiencies, as well as their specificities, analyzing their genotoxic off-target activities is important. Thus, we have developed a protocol that follows the fate of a biotin-labeled single-stranded oligodeoxynucleotide in human cells based on its physical incorporation into the targeted genome. Affected chromosomal fragments are enriched and preferably sequenced by nanopore sequencing. This protocol was validated in gene repair experiments without intentionally inducing a DNA double-strand break. For a 21-nucleotide-long phosphorothioate-modified oligodeoxynucleotide, we compiled a broad array of error-free incorporations, point mutations, indels, and structural rearrangements from actively dividing HEK293-derived cells. Additionally, we demonstrated the usefulness of this approach for primary cells by treating human CD34(+) hematopoietic stem and progenitor cells with a 100-nucleotide-long unmodified oligodeoxynucleotide directed against the endogenous CYBB locus. This work should pave the way for future genotoxicity analyses. Concerning genome engineering approaches based on nuclease-induced DNA double-strand breaks, this protocol could aid in detecting the unwanted effects caused by the donor fragments themselves.

Ramakrishna, S., et al. (2014). "Gene disruption by cell-penetrating peptide-mediated delivery of Cas9 protein and guide RNA." Genome Res **24**(6): 1020-1027.

RNA-guided endonucleases (RGENs) derived from the CRISPR/Cas system represent an efficient tool for genome editing. RGENs consist of two components: Cas9 protein and guide RNA. Plasmid-mediated delivery of these components into cells can result in uncontrolled integration of the plasmid sequence into the host genome, and unwanted immune responses and potential safety problems that can be caused by the bacterial sequences. Furthermore, this delivery method requires transfection tools. Here we show that simple treatment with cell-penetrating peptide (CPP)-conjugated recombinant Cas9 protein and CPP-complexed guide RNAs leads to endogenous gene disruptions in human cell lines. The Cas9 protein was conjugated to CPP via a thioether bond, whereas the guide RNA was complexed with CPP, forming condensed, positively charged nanoparticles. Simultaneous and sequential treatment of human cells, including embryonic stem cells, dermal fibroblasts, HEK293T cells, HeLa cells, and embryonic carcinoma cells, with the modified Cas9 and guide RNA, leads to efficient gene disruptions with reduced off-target mutations relative to plasmid transfections, resulting in the generation of clones containing RGEN-induced mutations. Our CPP-mediated RGEN delivery process provides a plasmid-free and additional transfection reagent-free method to use this tool with reduced off-target effects. We envision that our method will facilitate RGEN-directed genome editing.

Reilly, S. K. and J. P. Noonan (2016). "Evolution of Gene Regulation in Humans." Annu Rev Genomics Hum Genet **17**: 45-67.

As a species, we possess unique biological features that distinguish us from other primates. Here, we review recent efforts to identify changes in gene regulation that drove the evolution of novel human phenotypes. We discuss genotype-directed comparisons of human and nonhuman primate genomes to identify human-specific genetic changes that may encode new regulatory functions. We also review phenotype-directed approaches, which use comparisons of gene expression or regulatory function in homologous human and nonhuman primate cells and tissues to identify changes in expression levels or regulatory activity that may be due to genetic changes in humans. Together, these studies are beginning to reveal the landscape of regulatory innovation in human evolution and point to specific regulatory changes for further study. Finally, we highlight two novel strategies to model human-specific regulatory functions in vivo: primate induced pluripotent stem cells and the generation of humanized mice by genome editing.

Roberts, B., et al. (2017). "Systematic gene tagging using CRISPR/Cas9 in human stem cells to illuminate cell organization." Mol Biol Cell **28**(21): 2854-2874.

We present a CRISPR/Cas9 genome-editing strategy to systematically tag endogenous proteins with fluorescent tags in human induced pluripotent stem cells (hiPSC). To date, we have generated multiple hiPSC lines with monoallelic green fluorescent protein tags labeling 10 proteins representing major cellular structures. The tagged proteins include alpha tubulin, beta actin, desmoplakin, fibrillarin, nuclear lamin B1, nonmuscle myosin heavy chain IIB, paxillin, Sec61 beta, tight junction protein

ZO1, and Tom20. Our genome-editing methodology using Cas9/crRNA ribonuclear protein and donor plasmid coelectroporation, followed by fluorescence-based enrichment of edited cells, typically resulted in <0.1-4% homology-directed repair (HDR). Twenty-five percent of clones generated from each edited population were precisely edited. Furthermore, 92% (36/39) of expanded clonal lines displayed robust morphology, genomic stability, expression and localization of the tagged protein to the appropriate subcellular structure, pluripotency-marker expression, and multilineage differentiation. It is our conclusion that, if cell lines are confirmed to harbor an appropriate gene edit, pluripotency, differentiation potential, and genomic stability are typically maintained during the clonal line-generation process. The data described here reveal general trends that emerged from this systematic gene-tagging approach. Final clonal lines corresponding to each of the 10 cellular structures are now available to the research community.

Rossant, J. (2018). "Gene editing in human development: ethical concerns and practical applications." Development **145**(16).

The amazing power of CRISPR-Cas9 gene editing tools and other related technologies has impacted all areas of biology today. It has also raised ethical concerns, particularly with regard to the possibility of generating heritable changes in the human genome - so-called germline gene editing. Although technical and safety issues suggest that this approach is far from clinical application, gene editing as a research tool is moving forward in human embryos, non-human primates and in stem cell-derived embryoids. These studies are already providing new information relevant to our understanding of normal human development, infertility, early pregnancy loss and pluripotent stem cell origins.

Rubio, A., et al. (2016). "Rapid and efficient CRISPR/Cas9 gene inactivation in human neurons during human pluripotent stem cell differentiation and direct reprogramming." Sci Rep **6**: 37540.

The CRISPR/Cas9 system is a rapid and customizable tool for gene editing in mammalian cells. In particular, this approach has widely opened new opportunities for genetic studies in neurological disease. Human neurons can be differentiated in vitro from hPSC (human Pluripotent Stem Cells), hNPCs (human Neural Precursor Cells) or even directly reprogrammed from fibroblasts. Here, we described a new platform which enables, rapid and efficient CRISPR/Cas9-mediated genome targeting simultaneously with three different paradigms for in vitro generation of neurons. This system was employed to inactivate two genes associated with neurological disorder (TSC2 and KCNQ2) and achieved up to 85% efficiency of gene targeting in the differentiated cells. In particular, we devised a protocol that, combining the expression of the CRISPR components with neurogenic factors, generated functional human neurons highly enriched for the desired genome modification in only 5 weeks. This new approach is easy, fast and that does not require the generation of stable isogenic clones, practice that is time consuming and for some genes not feasible.

Rutter, G. A. (2016). "Modeling Type 2 Diabetes GWAS Candidate Gene Function in hESCs." Cell Stem Cell **19**(3): 281-282.

Type 2 diabetes is a complex polygenic disorder that affects about 1 in 12 adults. In this issue of Cell Stem Cell, Zeng et al. (2016) elegantly combine CRISPR-based gene editing in hESCs with directed beta cell differentiation to investigate the functions of genes highlighted by genome-wide association studies (GWAS) for this disease.

Ryu, J., et al. (2018). "Use of gene-editing technology to introduce targeted modifications in pigs." J Anim Sci Biotechnol **9**: 5.

Pigs are an important resource in agriculture and serve as a model for human diseases. Due to their physiological and anatomical similarities with humans, pigs can recapitulate symptoms of human diseases, making them a useful model in biomedicine. However, in the past pig models have not been widely used partially because of the difficulty in genetic modification. The lack of true embryonic stem cells in pigs forced researchers to utilize genetic modification in somatic cells and somatic cell nuclear transfer (SCNT) to generate genetically engineered (GE) pigs carrying site-specific modifications. Although possible, this approach is extremely inefficient and GE pigs born through this method often presented developmental defects associated with the cloning process. Advancement in the gene-editing systems such as Zinc-Finger Nucleases (ZFNs), Transcription activator-like effector nucleases (TALENs), and the Clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated 9 (Cas9) system have dramatically increased the efficiency of producing GE pigs. These gene-editing systems, specifically engineered endonucleases, are based on inducing double-stranded breaks (DSBs) at a specific location, and then site-specific modifications can be introduced through one of the two DNA repair pathways: non-homologous end joining (NHEJ) or homology direct repair (HDR). Random insertions or deletions (indels) can be introduced through NHEJ and

specific nucleotide sequences can be introduced through HDR, if donor DNA is provided. Use of these engineered endonucleases provides a higher success in genetic modifications, multiallelic modification of the genome, and an opportunity to introduce site-specific modifications during embryogenesis, thus bypassing the need of SCNT in GE pig production. This review will provide a historical prospective of GE pig production and examples of how the gene-editing system, led by engineered endonucleases, have improved GE pig production. We will also present some of our current progress related to the optimal use of CRISPR/Cas9 system during embryogenesis.

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.

Santos, D. P., et al. (2016). "Comprehensive Protocols for CRISPR/Cas9-based Gene Editing in Human Pluripotent Stem Cells." Curr Protoc Stem Cell Biol **38**: 5B 6 1-5B 6 60.

Genome editing of human pluripotent stem cells (hPSCs) with the CRISPR/Cas9 system has the potential to revolutionize hPSC-based disease modeling, drug screening, and transplantation therapy. Here, we aim to provide a single resource to enable groups, even those with limited experience with hPSC culture or the CRISPR/Cas9 system, to successfully perform genome editing. The methods are presented in detail and are supported by a theoretical framework to allow for the incorporation of inevitable improvements in the rapidly evolving gene-editing field. We describe protocols to generate hPSC lines with gene-specific knock-outs, small targeted mutations, or knock-in reporters. (c) 2016 by John Wiley & Sons, Inc.

Schiroli, G., et al. (2017). "Preclinical modeling highlights the therapeutic potential of hematopoietic stem cell gene editing for correction of SCID-X1." Sci Transl Med **9**(411).

Targeted genome editing in hematopoietic stem/progenitor cells (HSPCs) is an attractive strategy for treating immunohematological diseases. However, the limited efficiency of homology-directed editing in primitive HSPCs constrains the yield of corrected cells and might affect the feasibility and safety of clinical translation. These concerns need to be addressed in stringent preclinical models and overcome by developing more efficient editing methods. We generated a humanized X-linked severe combined immunodeficiency (SCID-X1) mouse model and evaluated the efficacy and safety of hematopoietic reconstitution from limited input of functional HSPCs, establishing thresholds for full correction upon different types of conditioning. Unexpectedly, conditioning before HSPC infusion was required to protect the mice from lymphoma developing when transplanting small numbers of progenitors. We then designed a one-size-fits-all IL2RG (interleukin-2 receptor common gamma-chain) gene correction strategy and, using the same reagents suitable for correction of human HSPC, validated the edited human gene in the disease model in vivo, providing evidence of targeted gene editing in mouse HSPCs and demonstrating the functionality of the IL2RG-edited lymphoid progeny. Finally, we optimized editing reagents and protocol for human HSPCs and attained the threshold of IL2RG editing in long-term repopulating cells predicted to safely rescue the disease, using clinically relevant HSPC sources and highly specific zinc finger nucleases or CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR-associated protein 9). Overall, our work establishes the rationale and guiding principles for clinical translation of SCID-X1 gene editing and provides a framework for developing gene correction for other diseases.

Seah, Y. F., et al. (2015). "Induced Pluripotency and Gene Editing in Disease Modelling: Perspectives and Challenges." Int J Mol Sci **16**(12): 28614-28634.

Embryonic stem cells (ESCs) are chiefly characterized by their ability to self-renew and to differentiate into any cell type derived from the three

main germ layers. It was demonstrated that somatic cells could be reprogrammed to form induced pluripotent stem cells (iPSCs) via various strategies. Gene editing is a technique that can be used to make targeted changes in the genome, and the efficiency of this process has been significantly enhanced by recent advancements. The use of engineered endonucleases, such as homing endonucleases, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and Cas9 of the CRISPR system, has significantly enhanced the efficiency of gene editing. The combination of somatic cell reprogramming with gene editing enables us to model human diseases in vitro, in a manner considered superior to animal disease models. In this review, we discuss the various strategies of reprogramming and gene targeting with an emphasis on the current advancements and challenges of using these techniques to model human diseases.

Soldner, F., et al. (2016). "Parkinson-associated risk variant in distal enhancer of alpha-synuclein modulates target gene expression." Nature **533**(7601): 95-99.

Genome-wide association studies (GWAS) have identified numerous genetic variants associated with complex diseases, but mechanistic insights are impeded by a lack of understanding of how specific risk variants functionally contribute to the underlying pathogenesis. It has been proposed that cis-acting effects of non-coding risk variants on gene expression are a major factor for phenotypic variation of complex traits and disease susceptibility. Recent genome-scale epigenetic studies have highlighted the enrichment of GWAS-identified variants in regulatory DNA elements of disease-relevant cell types. Furthermore, single nucleotide polymorphism (SNP)-specific changes in transcription factor binding are correlated with heritable alterations in chromatin state and considered a major mediator of sequence-dependent regulation of gene expression. Here we describe a novel strategy to functionally dissect the cis-acting effect of genetic risk variants in regulatory elements on gene expression by combining genome-wide epigenetic information with clustered regularly-interspaced short palindromic repeats (CRISPR)/Cas9 genome editing in human pluripotent stem cells. By generating a genetically precisely controlled experimental system, we identify a common Parkinson's disease associated risk variant in a non-coding distal enhancer element that regulates the expression of alpha-synuclein (SNCA), a key gene implicated in the pathogenesis of Parkinson's disease. Our data suggest that the transcriptional deregulation of SNCA is associated with sequence-dependent binding of the brain-specific transcription factors EMX2 and NKX6-1. This work establishes an experimental paradigm to functionally connect genetic variation with disease-relevant phenotypes.

Sun, N. and H. Zhao (2014). "Seamless correction of the sickle cell disease mutation of the HBB gene in human induced pluripotent stem cells using TALENs." Biotechnol Bioeng **111**(5): 1048-1053.

Sickle cell disease (SCD) is the most common human genetic disease which is caused by a single mutation of human beta-globin (HBB) gene. The lack of long-term treatment makes the development of reliable cell and gene therapies highly desirable. Disease-specific patient-derived human induced pluripotent stem cells (hiPSCs) have great potential for developing novel cell and gene therapies. With the disease-causing mutations corrected in situ, patient-derived hiPSCs can restore normal cell functions and serve as a renewable autologous cell source for the treatment of genetic disorders. Here we successfully utilized transcription activator-like effector nucleases (TALENs), a recently emerged novel genome editing tool, to correct the SCD mutation in patient-derived hiPSCs. The TALENs we have engineered are highly specific and generate minimal off-target effects. In combination with piggyBac transposon, TALEN-mediated gene targeting leaves no residual ectopic sequences at the site of correction and the corrected hiPSCs retain full pluripotency and a normal karyotype. Our study demonstrates an important first step of using TALENs for the treatment of genetic diseases such as SCD, which represents a significant advance toward hiPSC-based cell and gene therapies.

Suzuki, K., et al. (2014). "Targeted gene correction minimally impacts whole-genome mutational load in human-disease-specific induced pluripotent stem cell clones." Cell Stem Cell **15**(1): 31-36.

The utility of genome editing technologies for disease modeling and developing cellular therapies has been extensively documented, but the impact of these technologies on mutational load at the whole-genome level remains unclear. We performed whole-genome sequencing to evaluate the mutational load at single-base resolution in individual gene-corrected human induced pluripotent stem cell (hiPSC) clones in three different disease models. In single-cell clones, gene correction by helper-dependent adenoviral vector (HDAdV) or Transcription Activator-Like Effector Nuclease (TALEN) exhibited few off-target effects and a low level of sequence variation, comparable to that accumulated in routine hiPSC culture. The sequence variants were randomly distributed and unique to individual clones. We also combined both

technologies and developed a TALEN-HDAdV hybrid vector, which significantly increased gene-correction efficiency in hiPSCs. Therefore, with careful monitoring via whole-genome sequencing it is possible to apply genome editing to human pluripotent cells with minimal impact on genomic mutational load.

Tagliafierro, L. and O. Chiba-Falek (2016). "Up-regulation of SNCA gene expression: implications to synucleinopathies." Neurogenetics **17**(3): 145-157.

Synucleinopathies are a group of neurodegenerative diseases that share a common pathological lesion of intracellular protein inclusions largely composed by aggregates of alpha-synuclein protein. Accumulating evidence, including genome wide association studies, has implicated alpha-synuclein (SNCA) gene in the etiology of synucleinopathies. However, the precise variants within SNCA gene that contribute to the sporadic forms of Parkinson's disease (PD), dementia with Lewy bodies (DLB), multiple system atrophy (MSA), and other synucleinopathies and their molecular mechanisms of action remain elusive. It has been suggested that SNCA expression levels are critical for the development of these diseases. Here, we review several model systems that have been developed to advance the understanding of the role of SNCA expression levels in the etiology of synucleinopathies. We also describe different molecular mechanisms that regulate SNCA gene expression and discuss possible strategies for SNCA down-regulation as means for therapeutic approaches. Finally, we highlight some examples that underscore the relationships between the genetic association findings and the regulatory mechanisms of SNCA expression, which suggest that genetic variability in SNCA locus is directly responsible, at least in part, to the changes in gene expression and explain the reported associations of SNCA with synucleinopathies. Future studies utilizing induced pluripotent stem cells (iPSCs)-derived neuronal lines and genome editing by CRISPR/Cas9, will allow us to validate, characterize, and manipulate the effects of particular cis-genetic variants on SNCA expression. Moreover, this model system will enable us to compare different neuronal and glial lineages involved in synucleinopathies representing an attractive strategy to elucidate-common and specific-SNCA-genetic variants, regulatory mechanisms, and vulnerable expression levels underlying synucleinopathy spectrum disorders. This forthcoming knowledge will support the development of precision medicine for synucleinopathies.

Tolmachov, O. E. (2015). "Transgenic DNA modules with pre-programmed self-destruction: Universal molecular devices to escape 'genetic litter' in gene and cell therapy." Med Hypotheses **85**(5): 686-689.

Gene delivery to human somatic cells is a well-established therapeutic strategy to treat a variety of diseases. In addition, gene transfer to human cells is required to generate human induced pluripotent cells and also to eliminate tumorigenic undifferentiated cells in many types of stem-cell derived transplantation material. The expression of transgenes in these medical technologies is often required only in some of the recipient cells and only in specific limited time-windows, with inappropriately located or untimely expressed transgenes presenting a risk of undesired collateral effects. Unfortunately, current gene transfer procedures commonly result in a number of cells in the patient's body containing fragments of transferred genetic material which are either not therapeutically necessary at all, are no longer necessary or are necessary but in some other cells. Such transgenic material in the patient, created as a by-product of the chosen therapeutic procedure, constitutes, in fact, 'genetic litter', that is, persisting potentially-hazardous foreign genetic material which is neither required therapeutically nor explicitly chosen by an informed and free-willing person as an artificial body element. Wider use and more frequent administration of gene and cell therapy in the future are likely to give greater prominence to the issue of misdelivered genetic medicines and of their unwanted remainders accumulating in human bodies. Thus, novel DNA templates, which, on the one hand, are capable of providing transgene expression over broad time-windows, and, on the other hand, do not leave unwanted permanent 'genetic traces', are required. I propose that the problem of 'genetic litter' in patients' bodies can be addressed through the employment of a new type of gene vectors delivering DNA-based transgenic modules with pre-programmed self-destruction. Such vectors could deliver therapeutic DNA cargo and then execute self-liquidation through pre-scheduled activation of co-delivered genome editing tools, such as CRISPR/Cas9 nucleases, specific for the DNA to be eliminated. In this model, all unnecessary transgenic DNA is edited away precisely at a desired time point. Activity of the gene correction apparatus for the specific and effective destruction of transgenic DNA could be turned on by well-timed external signals or could be triggered through intracellular sensors of particular epigenetic signatures. It is expected that the employment of the proposed DNA-based gene vectors equipped with a transgene self-destruct mechanism can extend the safe and ethical application of gene and cell therapy to a broader range of curative and lifestyle-choice medical treatments, e.g., full body prophylactic gene therapy of cancer.

Vakulskas, C. A., et al. (2018). "A high-fidelity Cas9 mutant delivered as a ribonucleoprotein complex enables efficient gene editing in human hematopoietic stem and progenitor cells." Nat Med **24**(8): 1216-1224.

Translation of the CRISPR-Cas9 system to human therapeutics holds high promise. However, specificity remains a concern especially when modifying stem cell populations. We show that existing rationally engineered Cas9 high-fidelity variants have reduced on-target activity when using the therapeutically relevant ribonucleoprotein (RNP) delivery method. Therefore, we devised an unbiased bacterial screen to isolate variants that retain activity in the RNP format. Introduction of a single point mutation, p.R691A, in Cas9 (high-fidelity (HiFi) Cas9) retained the high on-target activity of Cas9 while reducing off-target editing. HiFi Cas9 induces robust AAV6-mediated gene targeting at five therapeutically relevant loci (HBB, IL2RG, CCR5, HEXB, and TRAC) in human CD34(+) hematopoietic stem and progenitor cells (HSPCs) as well as primary T cells. We also show that HiFi Cas9 mediates high-level correction of the sickle cell disease (SCD)-causing p.E6V mutation in HSPCs derived from patients with SCD. We anticipate that HiFi Cas9 will have wide utility for both basic science and therapeutic genome-editing applications.

Wang, Y., et al. (2017). "Integration-defective lentiviral vector mediates efficient gene editing through homology-directed repair in human embryonic stem cells." Nucleic Acids Res **45**(5): e29.

Human embryonic stem cells (hESCs) are used as platforms for disease study, drug screening and cell-based therapy. To facilitate these applications, it is frequently necessary to genetically manipulate the hESC genome. Gene editing with engineered nucleases enables site-specific genetic modification of the human genome through homology-directed repair (HDR). However, the frequency of HDR remains low in hESCs. We combined efficient expression of engineered nucleases and integration-defective lentiviral vector (IDLV) transduction for donor template delivery to mediate HDR in hESC line WA09. This strategy led to highly efficient HDR with more than 80% of the selected WA09 clones harboring the transgene inserted at the targeted genomic locus. However, certain portions of the HDR clones contained the concatemeric IDLV genomic structure at the target site, probably resulted from recombination of the IDLV genomic input before HDR with the target. We found that the integrase protein of IDLV mediated the highly efficient HDR through the recruitment of a cellular protein, LEDGF/p75. This study demonstrates that IDLV-mediated HDR is a powerful and broadly applicable technology to carry out site-specific gene modification in hESCs.

Worsdorfer, P., et al. (2018). "The role of connexins during early embryonic development: pluripotent stem cells, gene editing, and artificial embryonic tissues as tools to close the knowledge gap." Histochem Cell Biol **150**(4): 327-339.

Since almost 4 decades, connexins have been discussed as important regulators of embryogenesis. Several different members of the gene family can be detected in the preimplantation embryo and during gastrulation. However, genetically engineered mice deficient for every connexin expressed during early development are available and even double-deficient mice were generated. Interestingly, all of these mice complete gastrulation without any abnormalities. This raises the question if the role of connexins has been overrated or if other gene family members compensate and mask their importance. To answer this question, embryos completely devoid of any gap junctional communication need to be investigated. This is challenging because a variety of connexin genes are co-expressed and some null mutations lead to a lethal phenotype. In addition, maternal connexin transcripts were described to persist until the blastocyst stage. In this review, we summarize the current knowledge about the role of connexins during preimplantation development and in embryonic stem cells. We propose that the use of pluripotent stem cells, trophoblast stem cells, as well as artificial embryo-like structures and organoid cultures in combination with multiplex CRISPR/Cas9-based genome editing provides a powerful platform to comprehensively readdress this issue and decipher the role of connexins during lineage decision, differentiation, and morphogenesis in a cell culture model for mouse and human development.

Wu, M., et al. (2018). "Conditional gene knockout and reconstitution in human iPSCs with an inducible Cas9 system." Stem Cell Res **29**: 6-14.

Precise genome editing in human induced pluripotent stem cells (iPSCs) significantly enhances our capability to use human iPSCs for disease modeling, drug testing and screening as well as investigation of human cell biology. In this study, we seek to achieve conditional expression of the CD55 gene in order to interrogate its functions. We used two human iPSC lines that have unique genotypes, and constructed an inducible Cas9 gene expression system that is integrated at the AAVS1 safe harbor site in the human genome. Using paired guide RNAs, we observed efficient knock-out with an intended deletion in the coding region of several genes including CD55 and ETV6 genes. This paired guide RNA approach enabled us to efficiently identify homozygous iPSC

clones with an intended deletion. Once an iPSC clone lacking CD55 expression was identified and characterized, we were able to use the same doxycycline system to induce expression of a CD55 transgene from a piggyBac vector, in both undifferentiated and differentiated iPSCs. This single cell line of gene knock-out complemented with an inducible transgene is sufficient to achieve conditional expression of the CD55 gene. The methodology described here is broadly applicable to other genes in order to interrogate their functions.

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.

Xiao, D., et al. (2016). "A novel luciferase knock-in reporter system for studying transcriptional regulation of the human Sox2 gene." J Biotechnol **219**: 110-116.

Sox2 is an important transcriptional factor that has multiple functions in stem cell maintenance and tumorigenesis. To investigate the transcriptional regulation of the Sox2 gene, a luciferase knock-in reporter system was established in HEK293 cells by placing the luciferase gene in the genome under the control of the Sox2 gene promoter using a transcription activator-like effector nuclease (TALEN)-mediated genome editing technique. PCR and Southern blot results confirmed the site-specific integration of a single copy of the exogenous luciferase gene into the genome. To prove the reliability and sensitivity of this novel luciferase knock-in system, a CRISPR/Cas transcription activation system for the Sox2 gene was constructed and applied to the knock-in system. The results indicated that luciferase activity was directly correlated with the activity of the Sox2 endogenous promoter. This novel system will be a useful tool to study the transcriptional regulation of Sox2, and has great potential in medical and industrial applications.

Xu, X., et al. (2018). "Efficient homology-directed gene editing by CRISPR/Cas9 in human stem and primary cells using tube electroporation." Sci Rep **8**(1): 11649.

CRISPR/Cas9 efficiently generates gene knock-out via nonhomologous end joining (NHEJ), but the efficiency of precise homology-directed repair (HDR) is substantially lower, especially in the hard-to-transfect human stem cells and primary cells. Herein we report a tube electroporation method that can effectively transfect human stem cells and primary cells with minimal cytotoxicity. When applied to genome editing using CRISPR/Cas9 along with single stranded DNA oligonucleotide (ssODN) template in human induced pluripotent stem cells (iPSCs), up to 42.1% HDR rate was achieved, drastically higher than many reported before. We demonstrated that the high HDR efficiency can be utilized to increase the gene ablation rate in cells relevant to clinical applications, by knocking-out beta2-microglobulin (B2M) in primary human mesenchymal stem cells (MSCs, 37.3% to 80.2%), and programmed death-1 (PD-1) in primary human T cells (42.6% to 58.6%). Given the generality and efficiency, we expect that the method will have immediate impacts in cell research as well as immuno- and transplantation therapies.

Xu, X., et al. (2017). "Reversal of Phenotypic Abnormalities by CRISPR/Cas9-Mediated Gene Correction in Huntington Disease Patient-Derived Induced Pluripotent Stem Cells." Stem Cell Reports **8**(3): 619-633.

Huntington disease (HD) is a dominant neurodegenerative disorder caused by a CAG repeat expansion in HTT. Here we report correction of HD human induced pluripotent stem cells (hiPSCs) using a CRISPR-Cas9 and piggyBac transposon-based approach. We show that both HD and corrected isogenic hiPSCs can be differentiated into excitable, synaptically active forebrain neurons. We further demonstrate that phenotypic abnormalities in HD hiPSC-derived neural cells, including impaired neural rosette formation, increased susceptibility to growth factor withdrawal, and deficits in mitochondrial respiration, are rescued in isogenic controls. Importantly, using genome-wide expression analysis, we show that a number of apparent gene expression differences detected between HD and non-related healthy control lines are absent between HD and corrected lines, suggesting that these differences are likely related to genetic background rather than HD-specific effects. Our study demonstrates correction of HD hiPSCs and associated phenotypic abnormalities, and the importance of isogenic controls for disease modeling using hiPSCs.

Yada, R. C., et al. (2017). "CRISPR/Cas9-Based Safe-Harbor Gene Editing in Rhesus iPSCs." Curr Protoc Stem Cell Biol **43**: 5A 11 11-15A 11 14.

NHP iPSCs provide a unique opportunity to test safety and efficacy of iPSC-derived therapies in clinically relevant NHP models. To monitor these cells in vivo, there is a need for safe and efficient labeling methods. Gene insertion into genomic safe harbors (GSHs) supports reliable transgene expression while minimizing the risk the modification poses to the host genome or target cell. Specifically, this protocol demonstrates targeting of the adeno-associated virus site 1 (AAVS1), one of the most widely used GSH loci in the human genome, with CRISPR/Cas9, allowing targeted marker or therapeutic gene insertion in rhesus macaque induced pluripotent stem cells (RhiPSCs). Furthermore, detailed instructions for screening targeted clones and a tool for assessing potential off-target nuclease activity are provided. (c) 2017 by John Wiley & Sons, Inc.

Yamamoto, S., et al. (2015). "Efficient gene-targeting in rat embryonic stem cells by CRISPR/Cas and generation of human kynurenine aminotransferase II (KAT II) knock-in rat." Transgenic Res **24**(6): 991-1001.

The relative proportion of kynurenine aminotransferase (KAT) I-IV activities in the brain is similar between humans and rats. Moreover, KAT II is considered to be the main enzyme for kynurenic acid production in the brain. Taken together, human KAT II knock-in (hKAT II KI) rats will become a valuable tool for the evaluation of KAT II targeted drugs as a human mimetic model. Although we initially tried the approach by conventional gene-targeting via embryonic stem cells (ESCs) to generate them, we had to give up the production because of no recombinant ESCs. Accordingly, we developed a method to improve the efficiency of homologous recombination (HR) in ESCs by the combination with the CRISPR/Cas system. Co-electroporation of Cas9 plasmid, single guide RNA plasmid and hKAT II KI vector increased the number of drug-resistant colonies and greatly enhanced the HR efficiency from 0 to 36 %. All the clones which we obtained showed the same sequence as designed. These recombinant clones resulted in chimeras that transmitted the hKAT II KI allele to their offspring. hKAT II KI rats showed no reduction of KATs mRNA expression and the amount of kynurenic acid was similar between the hKAT II KI rats and the wild type in their brains. These results indicate that the methodology presented in this report can overcome the problem encountered in conventional gene-targeting that prevented production of humanized rats.

Yang, Y., et al. (2016). "Naive Induced Pluripotent Stem Cells Generated From beta-Thalassemia Fibroblasts Allow Efficient Gene Correction With CRISPR/Cas9." Stem Cells Transl Med **5**(1): 8-19.

UNLABELLED: Conventional primed human embryonic stem cells and induced pluripotent stem cells (iPSCs) exhibit molecular and biological characteristics distinct from pluripotent stem cells in the naive state. Although naive pluripotent stem cells show much higher levels of self-renewal ability and multidifferentiation capacity, it is unknown whether naive iPSCs can be generated directly from patient somatic cells and will be superior to primed iPSCs. In the present study, we used an established 5i/L/FA system to directly reprogram fibroblasts of a patient with beta-thalassemia into transgene-free naive iPSCs with molecular signatures of ground-state pluripotency. Furthermore, these naive iPSCs can efficiently produce cross-species chimeras. Importantly, using the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 nuclease genome editing system, these naive iPSCs exhibit significantly improved gene-correction efficiencies compared with the corresponding primed iPSCs. Furthermore, human naive iPSCs could be directly generated from noninvasively collected urinary cells, which are easily acquired and thus represent an excellent cell resource for further clinical trials. Therefore, our findings demonstrate the feasibility and superiority of using patient-specific iPSCs in the naive state for disease modeling, gene

editing, and future clinical therapy. SIGNIFICANCE: In the present study, transgene-free naive induced pluripotent stem cells (iPSCs) directly converted from the fibroblasts of a patient with beta-thalassemia in a defined culture system were generated. These naive iPSCs, which show ground-state pluripotency, exhibited significantly improved single-cell cloning ability, recovery capacity, and gene-targeting efficiency compared with conventional primed iPSCs. These results provide an improved strategy for personalized treatment of genetic diseases such as beta-thalassemia.

Yates, F. and G. Q. Daley (2006). "Progress and prospects: gene transfer into embryonic stem cells." Gene Ther **13**(20): 1431-1439.

With the isolation of human embryonic stem cells (hESCs) in 1998 came the realization of a long-sought aspiration for an unlimited source of human tissue. The difficulty of differentiating ESCs to pure, clinically exploitable cell populations to treat genetic and degenerative diseases is being solved in part with the help of genetically modified cell lines. With progress in genome editing and somatic cell nuclear transfer, it is theoretically possible to obtain genetically repaired isogenic cells. Moreover, the prospect of being able to select, isolate and expand a single cell to a vast population of cells could achieve a unique level of quality control, until now unattainable in the field of gene therapy. Most of the tools necessary to develop these strategies already exist in the mouse ESC system. We review here the advances accomplished in those fields and present some possible applications to hESC research.

Yoshida, T., et al. (2014). "The use of induced pluripotent stem cells to reveal pathogenic gene mutations and explore treatments for retinitis pigmentosa." Mol Brain **7**: 45.

BACKGROUND: Retinitis pigmentosa (RP) is an inherited human retinal disorder that causes progressive photoreceptor cell loss, leading to severe vision impairment or blindness. However, no effective therapy has been established to date. Although genetic mutations have been identified, the available clinical data are not always sufficient to elucidate the roles of these mutations in disease pathogenesis, a situation that is partially due to differences in genetic backgrounds. RESULTS: We generated induced pluripotent stem cells (iPSCs) from an RP patient carrying a rhodopsin mutation (E181K). Using helper-dependent adenoviral vector (HDAdV) gene transfer, the mutation was corrected in the patient's iPSCs and also introduced into control iPSCs. The cells were then subjected to retinal differentiation; the resulting rod photoreceptor cells were labeled with an Nrl promoter-driven enhanced green fluorescent protein (EGFP)-carrying adenovirus and purified using flow cytometry after 5 weeks of culture. Using this approach, we found a reduced survival rate in the photoreceptor cells with the E181K mutation, which was correlated with the increased expression of endoplasmic reticulum (ER) stress and apoptotic markers. The screening of therapeutic reagents showed that rapamycin, PP242, AICAR, NQDI-1, and salubrinal promoted the survival of the patient's iPSC-derived photoreceptor cells, with a concomitant reduction in markers of ER stress and apoptosis. Additionally, autophagy markers were found to be correlated with ER stress, suggesting that autophagy was reduced by suppressing ER stress-induced apoptotic changes. CONCLUSION: The use of RP patient-derived iPSCs combined with genome editing provided a versatile cellular system with which to define the roles of genetic mutations in isogenic iPSCs with or without mutation and also provided a system that can be used to explore candidate therapeutic approaches.

Yu, J., et al. (2016). "CHES-1-like, the ortholog of a non-obstructive azoospermia-associated gene, blocks germline stem cell differentiation by upregulating Dpp expression in Drosophila testis." Oncotarget **7**(27): 42303-42313.

Azoospermia is a high risk factor for testicular germ cell tumors, whose underlying molecular mechanisms remain unknown. In a genome-wide association study to identify novel loci associated with human non-obstructive azoospermia (NOA), we uncovered a single nucleotide polymorphism (rs1887102, P=2.60 x10-7) in a human gene FOXN3. FOXN3 is an evolutionarily conserved gene. We used Drosophila melanogaster as a model system to test whether CHES-1-like, the Drosophila FOXN3 ortholog, is required for male fertility. CHES-1-like knockout flies are viable and fertile, and show no defects in spermatogenesis. However, ectopic expression of CHES-1-like in germ cells significantly reduced male fertility. With CHES-1-like overexpression, spermatogonia fail to differentiate after four rounds of mitotic division, but continue to divide to form tumor like structures. In these testes, expression levels of differentiation factor, Bam, were reduced, but the expression region of Bam was expanded. Further reduced Bam expression in CHES-1-like expressing testes exhibited enhanced tumor-like structure formation. The expression of daughters against dpp (dad), a downstream gene of dpp signaling, was upregulated by CHES-1-like expression in testes. We found that CHES-1-like could directly bind to the dpp promoter. We propose a model that CHES-1-like overexpression in germ cells activates dpp expression,

inhibits spermatocyte differentiation, and finally leads to germ cell tumors.

Yu, K. R., et al. (2016). "Gene Editing of Human Hematopoietic Stem and Progenitor Cells: Promise and Potential Hurdles." Hum Gene Ther **27**(10): 729-740.

Hematopoietic stem and progenitor cells (HSPCs) have great therapeutic potential because of their ability to both self-renew and differentiate. It has been proposed that, given their unique properties, a small number of genetically modified HSPCs could accomplish lifelong, corrective reconstitution of the entire hematopoietic system in patients with various hematologic disorders. Scientists have demonstrated that gene addition therapies-targeted to HSPCs and using integrating retroviral vectors-possess clear clinical benefits in multiple diseases, among them immunodeficiencies, storage disorders, and hemoglobinopathies. Scientists attempting to develop clinically relevant gene therapy protocols have, however, encountered a number of unexpected hurdles because of their incomplete knowledge of target cells, genomic control, and gene transfer technologies. Targeted gene-editing technologies using engineered nucleases such as ZFN, TALEN, and/or CRISPR/Cas9 RGEN show great clinical promise, allowing for the site-specific correction of disease-causing mutations-a process with important applications in autosomal dominant or dominant-negative genetic disorders. The relative simplicity of the CRISPR/Cas9 system, in particular, has sparked an exponential increase in the scientific community's interest in and use of these gene-editing technologies. In this minireview, we discuss the specific applications of gene-editing technologies in human HSPCs, as informed by prior experience with gene addition strategies. HSPCs are desirable but challenging targets; the specific mechanisms these cells evolved to protect themselves from DNA damage render them potentially more susceptible to oncogenesis, especially given their ability to self-renew and their long-term proliferative potential. We further review scientists' experience with gene-editing technologies to date, focusing on strategies to move these techniques toward implementation in safe and effective clinical trials.

Yumlu, S., et al. (2017). "Gene editing and clonal isolation of human induced pluripotent stem cells using CRISPR/Cas9." Methods **121-122**: 29-44.

Human induced pluripotent stem cells (hiPSCs) represent an ideal in vitro platform to study human genetics and biology. The recent advent of programmable nucleases makes also the human genome amenable to experimental genetics through either the correction of mutations in patient-derived iPSC lines or the de novo introduction of mutations into otherwise healthy iPSCs. The production of specific and sometimes complex genotypes in multiple cell lines requires efficient and streamlined gene editing technologies. In this article we provide protocols for gene editing in hiPSCs. We presently achieve high rates of gene editing at up to three loci using a modified iCRISPR system. This system includes a doxycycline inducible Cas9 and sgRNA/reporter plasmids for the enrichment of transfected cells by fluorescence-activated cell sorting (FACS). Here we cover the selection of target sites, vector construction, transfection, and isolation and genotyping of modified hiPSC clones.

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.

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