CRISPR and Stem Cell Research Literatures

Mark Herbert, PhD

39-06 Main Street, Flushing, Queens, New York 11354, USA
ma8080@gmail.com

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. CRISPR (clustered regularly interspaced short palindromic repeats) is a family of DNA sequences found within the genomes of prokaryotic organisms such as bacteria and archaea. These sequences are derived from DNA fragments from viruses that have previously infected the prokaryote and are used to detect and destroy DNA from similar viruses during subsequent infections. Hence these sequences play a key role in the antiviral defense system of prokaryotes. Cas9 is an enzyme that uses CRISPR sequences as a guide to recognize and cleave specific strands of DNA that are complementary to the CRISPR sequence. Cas9 enzymes together with CRISPR sequences form the basis of a technology known as CRISPR/Cas9 that can be used to edit genes within organisms. This type of gene editing process has a wide variety of applications including use as a basic biology research tool, development of biotechnology products, and potentially to treat diseases. The CRISPR/Cas system is a prokaryotic immune system that confers resistance to foreign genetic elements such as those present within plasmids and phages that provides a form of acquired immunity. RNA harboring the spacer sequence helps Cas proteins recognize and cut foreign pathogenic DNA. Other RNA-guided Cas proteins cut foreign RNA. CRISPR are found in approximately 50% of sequenced bacterial genomes and nearly 90% of sequenced archaea. This article introduces recent research reports as references in the related studies.
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Key words: stem cell; CRISPR; Cas9; 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. CRISPR (clustered regularly interspaced short palindromic repeats) is a family of DNA sequences found within the genomes of prokaryotic organisms such as bacteria and archaea. These sequences are derived from DNA fragments from viruses that have previously infected the prokaryote and are used to detect and destroy DNA from similar viruses during subsequent infections. Hence these sequences play a key role in the antiviral defense system of prokaryotes. Cas9 is an enzyme that uses CRISPR sequences as a guide to recognize and cleave specific strands of DNA that are complementary to the CRISPR sequence. Cas9 enzymes together with CRISPR sequences form the basis of a technology known as CRISPR/Cas9 that can be used to edit genes within organisms. This type of gene editing process has a wide variety of applications including use as a basic biology research tool, development of biotechnology products, and potentially to treat diseases. The CRISPR/Cas system is a prokaryotic immune system that confers resistance to foreign genetic elements such as those present within plasmids and phages that provides a form of acquired immunity. RNA harboring the spacer sequence helps Cas proteins recognize and cut foreign pathogenic DNA. Other RNA-guided Cas proteins cut foreign RNA. CRISPR are found in approximately 50% of sequenced bacterial genomes and nearly 90% of sequenced archaea. This article introduces recent research reports as references in the related studies.

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Anderson, R. H., et al. (2018). "Generation of a CLTA reporter human induced pluripotent stem cell

line, CRMi001-A-1, using the CRISPR/Cas9 system to monitor endogenous clathrin trafficking." Stem Cell Res **33**: 95-99.

The most highly studied endocytic pathway, clathrin-dependent endocytosis, mediates a wide range of fundamental processes including nutrient internalization, receptor recycling, and signal transduction. In order to model tissue specific and developmental aspects of this process, CRISPR/Cas9 genomic editing was utilized to fluorescently label the C-terminus of clathrin light chain A (CLTA) within the phenotypically normal, parental CRMi001-A human induced pluripotent stem cell line. Successfully edited cells were isolated by fluorescently activated cell sorting, remained karyotypically normal, and maintained their differentiation potential. This cell line facilitates imaging of endogenous clathrin trafficking within varied cell types.

Balboa, D., et al. (2017). "Generation of a SOX2 reporter human induced pluripotent stem cell line using CRISPR/SaCas9." Stem Cell Res **22**: 16-19.

SOX2 is an important transcription factor involved in pluripotency maintenance, pluripotent reprogramming and differentiation towards neural lineages. Here we engineered the previously described HEL24.3 hiPSC to generate a SOX2 reporter by knocking-in a T2A fused nuclear tdTomato reporter cassette before the STOP codon of the SOX2 gene coding sequence. CRISPR/SaCas9-mediated stimulation of homologous recombination was utilized to facilitate faithful targeted insertion. This line accurately reports the expression of endogenous SOX2 and therefore constitutes a useful tool to study the SOX2 expression dynamics upon hiPSC culture, differentiation and somatic cell reprogramming.

Balboa, D., et al. (2017). "Generation of an OCT4 reporter human induced pluripotent stem cell line using CRISPR/SpCas9." Stem Cell Res **23**: 105-108.

OCT4 is a crucial transcription factor in the pluripotent stem cell gene regulatory network and an essential factor for pluripotent reprogramming. We engineered the previously reported HEL24.3 hiPSC to generate an OCT4 reporter cell line by knocking-in a T2A nuclear EmGFP reporter cassette before the OCT4 gene STOP codon sequence. To enhance targeted insertion, homologous recombination was stimulated using targeted cutting at the OCT4 STOP codon with CRISPR/SpCas9. This HEL24.3-OCT4-nEmGFP cell line faithfully reports endogenous OCT4 expression, serving as a useful tool to examine temporal changes in OCT4 expression in live cells during hiPSC culture, differentiation and somatic cell reprogramming.

Bara, A. M., et al. (2016). "Generation of a TLE3 heterozygous knockout human embryonic stem cell line using CRISPR-Cas9." Stem Cell Res **17**(2): 441-443.

Here, we generated a monoallelic mutation in the TLE3 (Transducin Like Enhancer of Split 3) gene using CRISPR-Cas9 editing in the human embryonic stem cell (hESC) line WA01. The heterozygous knockout cell line, TLE3-447-D08-A01, displays partial loss of TLE3 protein expression while maintaining pluripotency, differentiation potential and genomic integrity.

Ben Jehuda, R., et al. (2018). "CRISPR correction of the PRKAG2 gene mutation in the patient's induced pluripotent stem cell-derived cardiomyocytes eliminates electrophysiological and structural abnormalities." Heart Rhythm **15**(2): 267-276.

BACKGROUND: Mutations in the PRKAG2 gene encoding the gamma-subunit of adenosine monophosphate kinase (AMPK) cause hypertrophic cardiomyopathy (HCM) and familial Wolff-Parkinson-White (WPW) syndrome. Patients carrying the R302Q mutation in PRKAG2 present with sinus bradycardia, escape rhythms, ventricular preexcitation, supraventricular tachycardia, and atrioventricular block. This mutation affects AMPK activity and increases glycogen storage in cardiomyocytes. The link between glycogen storage, WPW syndrome, HCM, and arrhythmias remains unknown. OBJECTIVE: The purpose of this study was to investigate the pathological changes caused by the PRKAG2 mutation. We tested the hypothesis that patient's induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) display clinical aspects of the disease. METHODS: Using clustered regularly interspaced short palindromic repeats (CRISPR) technology, we corrected the mutation and then generated isogenic iPSC-CMs. Action potentials were recorded from spontaneously firing and paced cardiomyocytes using the patch clamp technique. Using a microelectrode array setup, we recorded electrograms from iPSC-CMs clusters. Transmission electron microscopy was used to detect ultrastructural abnormalities in the mutated iPSC-CMs. RESULTS: PRKAG2-mutated iPSC-CMs exhibited abnormal firing patterns, delayed afterdepolarizations, triggered arrhythmias, and augmented beat rate variability. Importantly, CRISPR correction eliminated the electrophysiological abnormalities, the augmented glycogen, storage, and cardiomyocyte hypertrophy. CONCLUSION: PRKAG2-mutated iPSC-CMs displayed functional and structural abnormalities,

which were abolished by correcting the mutation in the patient's iPSCs using CRISPR technology.

Chaterji, S., et al. (2017). "CRISPR Genome Engineering for Human Pluripotent Stem Cell Research." Theranostics **7**(18): 4445-4469.

The emergence of targeted and efficient genome editing technologies, such as repurposed bacterial programmable nucleases (e.g., CRISPR-Cas systems), has abetted the development of cell engineering approaches. Lessons learned from the development of RNA-interference (RNA-i) therapies can spur the translation of genome editing, such as those enabling the translation of human pluripotent stem cell engineering. In this review, we discuss the opportunities and the challenges of repurposing bacterial nucleases for genome editing, while appreciating their roles, primarily at the epigenomic granularity. First, we discuss the evolution of high-precision, genome editing technologies, highlighting CRISPR-Cas9. They exist in the form of programmable nucleases, engineered with sequence-specific localizing domains, and with the ability to revolutionize human stem cell technologies through precision targeting with greater on-target activities. Next, we highlight the major challenges that need to be met prior to bench-to-bedside translation, often learning from the path-to-clinic of complementary technologies, such as RNA-i. Finally, we suggest potential bioinformatics developments and CRISPR delivery vehicles that can be deployed to circumvent some of the challenges confronting genome editing technologies en route to the clinic.

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.

Christidi, E., et al. (2018). "CRISPR/Cas9-mediated genome editing in human stem cell-derived cardiomyocytes: Applications for cardiovascular disease modelling and cardiotoxicity screening." Drug Discov Today Technol **28**: 13-21.

Cardiovascular diseases (CVDs) are leading causes of death worldwide, and drug-induced cardiotoxicity is among the most common cause of drug withdrawal from the market. Improved models of cardiac tissue are needed to study the mechanisms of CVDs and drug-induced cardiotoxicity. Human pluripotent stem cell-derived cardiomyocytes (hPSC-CM) have provided a major advance to our ability to study these conditions. Combined with efficient genome editing technologies, such as CRISPR/Cas9, we now have the ability to study with greater resolution the genetic causes and underlying mechanisms of inherited and drug-induced cardiotoxicity, and to investigate new treatments. Here, we review recent advances in the use of hPSC-CMs and CRISPR/Cas9-mediated genome editing to study cardiotoxicity and model CVD.

Drost, J., et al. (2017). "Use of CRISPR-modified human stem cell organoids to study the origin of mutational signatures in cancer." Science **358**(6360): 234-238.

Mutational processes underlie cancer initiation and progression. Signatures of these processes in cancer genomes may explain cancer etiology and could hold diagnostic and prognostic value. We developed a strategy that can be used to explore the origin of cancer-associated mutational signatures. We used CRISPR-Cas9 technology to delete key DNA repair genes in human colon organoids, followed by delayed subcloning and whole-genome sequencing. We found that mutation accumulation in organoids deficient in the mismatch repair gene MLH1 is driven by replication errors and accurately models the mutation profiles observed in mismatch repair-deficient colorectal cancers. Application of this strategy to the cancer predisposition gene NTHL1, which encodes a base excision repair protein, revealed a mutational footprint (signature 30) previously observed in a breast cancer cohort. We show that signature 30 can arise from germline NTHL1 mutations.

Fei, J. F., et al. (2014). "CRISPR-mediated genomic deletion of Sox2 in the axolotl shows a requirement in spinal cord neural stem cell amplification during tail regeneration." Stem Cell Reports **3**(3): 444-459.

The salamander is the only tetrapod that functionally regenerates all cell types of the limb and spinal cord (SC) and thus represents an important regeneration model, but the lack of gene-knockout technology has limited molecular analysis. We compared transcriptional activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPRs) in the knockout of three loci in the axolotl and find that CRISPRs show highly penetrant knockout with less toxic effects compared to TALENs. Deletion of Sox2 in up to 100% of cells yielded viable F0 larvae with normal SC organization and ependymoglial cell marker expression such as GFAP and ZO-1. However, upon tail amputation, neural stem cell proliferation was inhibited, resulting in spinal-cord-specific regeneration failure. In contrast, the mesodermal blastema formed normally. Sox3 expression during development, but not regeneration, most likely allowed embryonic survival and the regeneration-specific phenotype. This analysis represents the first tissue-specific regeneration phenotype from the genomic deletion of a gene in the axolotl.

Gerace, D., et al. (2017). "CRISPR-targeted genome editing of mesenchymal stem cell-derived therapies for type 1 diabetes: a path to clinical success?" Stem Cell Res Ther **8**(1): 62.

Due to their ease of isolation, differentiation capabilities, and immunomodulatory properties, the therapeutic potential of mesenchymal stem cells (MSCs) has been assessed in numerous pre-clinical and clinical settings. Currently, whole pancreas or islet transplantation is the only cure for people with type 1 diabetes (T1D) and, due to the autoimmune nature of the disease, MSCs have been utilised either natively or transdifferentiated into insulin-producing cells (IPCs) as an alternative treatment. However, the initial success in pre-clinical animal models has not translated into successful clinical outcomes. Thus, this review will summarise the current state of MSC-derived therapies for the treatment of T1D in both the pre-clinical and clinical setting, in particular their use as an immunomodulatory therapy and targets for the generation of IPCs via gene modification. In this review, we highlight the limitations of current clinical trials of MSCs for the treatment of T1D, and suggest the novel clustered regularly interspaced short palindromic repeat (CRISPR) gene-editing technology and improved clinical trial design as strategies to translate pre-clinical success to the clinical setting.

Guo, D., et al. (2016). "Generation of an Abcc8 heterozygous mutation human embryonic stem cell line using CRISPR/Cas9." Stem Cell Res **17**(3): 670-672.

The gene of ATP-binding cassette subfamily C member 8 (Abcc8) is cytogenetically located at 11p15.1 and encodes the sulfonylurea receptor (SUR1). SUR1 is a subunit of ATP-sensitive potassium channel (KAPT) in the beta-cell regulating insulin secretion. Mutations of ABCC8 are responsible for congenital hyperinsulinism (CHI). Here we reported that an Abcc8 heterozygous mutant cell line was generated by CRISPR/Cas9 technique with 1bp insertion resulting in abnormal splicing on human embryonic stem cell line H1. The phenotypic characteristics of this cell line reveal defective KATP channel and diazoxide-responsive that provides ideal model for molecular pathology research and drug screening for CHI.

Guo, D., et al. (2016). "Generation of an Abcc8 homozygous mutation human embryonic stem cell line using CRISPR/Cas9." Stem Cell Res **17**(3): 640-642.

The gene of ATP-binding cassette subfamily C member 8 (Abcc8) is cytogenetically located at 11p15.1 and encodes the sulfonylurea receptor (SUR1). SUR1 is a subunit of ATP-sensitive potassium channel (KAPT) in the beta-cell regulating insulin secretion. Mutations of ABCC8 are responsible for congenital hyperinsulinism (CHI). Here we generated an Abcc8 homozygous mutant cell line by CRISPR/Cas9 technique with 22bp deletion resulting in abnormal splicing on human embryonic stem cell line H1. The phenotypic characteristics of this cell line reveal defective KATP channel and diazoxide-unresponsive that provides an ideal model for molecular pathology research and drug screening for CHI.

Herring, A., et al. (2016). "Generation of a TLE1 homozygous knockout human embryonic stem cell line using CRISPR-Cas9." Stem Cell Res **17**(2): 430-432.

Here, we generated a biallelic mutation in the TLE1 (Transducin Like Enhancer of Split 1) gene using CRISPR-Cas9 editing in the human embryonic stem cell (hESC) line WA01. The homozygous knockout cell line, TLE1-464-G04, displays loss of TLE1 protein expression while maintaining pluripotency, differentiation potential and genomic integrity.

Hunt, C. P. J., et al. (2017). "Characterising the developmental profile of human embryonic stem cell-derived medium spiny neuron progenitors and assessing mature neuron function using a CRISPR-generated human DARPP-32(WT/eGFP-AMP) reporter line." Neurochem Int **106**: 3-13.

In the developing ventral telencephalon, cells of the lateral ganglionic eminence (LGE) give rise to all medium spiny neurons (MSNs). This development occurs in response to a highly orchestrated series of

morphogenetic stimuli that pattern the resultant neurons as they develop. Striatal MSNs are characterised by expression of dopamine receptors, dopamine-and cyclic AMP-regulated phosphoprotein (DARPP32) and the neurotransmitter GABA. In this study, we demonstrate that fine tuning Wnt and hedgehog (SHH) signaling early in human embryonic stem cell differentiation can induce a subpallial progenitor molecular profile. Stimulation of TGFbeta signaling pathway by activin-A further supports patterning of progenitors to striatal precursors which adopt an LGE-specific gene signature. Moreover, we report that these MSNs also express markers associated with mature neuron function (cannabinoid, adenosine and dopamine receptors). To facilitate live-cell identification we generated a human embryonic stem cell line using CRISPR-mediated gene editing at the DARPP32 locus (DARPP32(WT/eGFP-AMP-LacZ)). The addition of dopamine to MSNs either increased, decreased or had no effect on intracellular calcium, indicating the presence of multiple dopamine receptor subtypes. In summary, we demonstrate greater control over early fate decisions using activin-A, Wnt and SHH to direct differentiation into MSNs. We also generate a DARPP32 reporter line that enables deeper pharmacological profiling and interrogation of complex receptor interactions in human MSNs.

Jayavaradhan, R., et al. (2019). "A Versatile Tool for the Quantification of CRISPR/Cas9-Induced Genome Editing Events in Human Hematopoietic Cell Lines and Hematopoietic Stem/Progenitor Cells." J Mol Biol **431**(1): 102-110.

The efficient site-specific DNA double-strand breaks (DSB) created by CRISPR/Cas9 has revolutionized genome engineering and has great potential for editing hematopoietic stem/progenitor cells (HSPCs). However, detailed understanding of the variables that influence choice of DNA-DSB repair (DDR) pathways by HSPC is required for therapeutic levels of editing in these clinically relevant cells. We developed a hematopoietic-reporter system that rapidly quantifies the three major DDR pathways utilized at the individual DSB created by CRISPR/Cas9-NHEJ, MMEJ, and HDR-and show its applicability in evaluating the different DDR outcomes utilized by human hematopoietic cell lines and primary human HSPC.

Jin, L. F. and J. S. Li (2016). "Generation of genetically modified mice using CRISPR/Cas9 and haploid embryonic stem cell systems." Dongwuxue Yanjiu **37**(4): 205-213.

With the development of high-throughput sequencing technology in the post-genomic era, researchers have concentrated their efforts on elucidating the relationships between genes and their corresponding functions. Recently, important progress has been achieved in the generation of genetically modified mice based on CRISPR/Cas9 and haploid embryonic stem cell (haESC) approaches, which provide new platforms for gene function analysis, human disease modeling, and gene therapy. Here, we review the CRISPR/Cas9 and haESC technology for the generation of genetically modified mice and discuss the key challenges in the application of these approaches.

Kim, H., et al. (2018). "Generation of a PXR reporter human induced pluripotent stem cell line (PXR-mCherry hiPSC) using the CRISPR/Cas9 system." Stem Cell Res **26**: 72-75.

Pregnane X receptor (PXR) is a key nuclear receptor that mediates drug metabolism and stimulates hepatocyte proliferation. However, the lack of PXR expression in human pluripotent stem cell-derived hepatocytes limits their application for drug screening and toxicity testing. Here, we generated a PXR-mCherry reporter human induced pluripotent stem cell (hiPSC) line using the CRISPR/Cas9 system. PXR-mCherry hiPSCs were pluripotent and had differentiation potential and a normal karyotype. This cell line is an important tool for identifying factors that increase PXR-mediated drug metabolism and hepatocyte proliferation.

Kim, S. J., et al. (2017). "Generation of a Nrf2 homozygous knockout human embryonic stem cell line using CRISPR/Cas9." Stem Cell Res **19**: 46-48.

Nuclear factor erythroid 2-related factor 2 (NFE2L2 or Nrf2) is a well-known transcription factor that regulates the expression of a large number of anti-oxidant genes in mammalian cells (J.H. Kim et al., 2014). Here, we generated a homozygous Nrf2 knockout human embryonic stem cell (hESC) line, H9Nrf2KO-A13, using the CRISPR/Cas9 genome editing method. The Nrf2 homozygous knockout H9 cell line maintains pluripotency, differentiation potential into three germ layers, and a normal karyotype.

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.

Leidy-Davis, T., et al. (2018). "Viable Mice with Extensive Gene Humanization (25-kbp) Created Using Embryonic Stem Cell/Blastocyst and CRISPR/Zygote Injection Approaches." Sci Rep **8**(1): 15028.

Here, we describe an expansion of the typical DNA size limitations associated with CRISPR knock-in technology, more specifically, the physical extent to which mouse genomic DNA can be replaced with donor (in this case, human) DNA at an orthologous locus by zygotic injection. Driving our efforts was the desire to create a whole animal model that would replace 17 kilobase pairs (kbp) of the mouse Bcl2l11 gene with the corresponding 25-kbp segment of human BCL2L11, including a conditionally removable segment (2.9-kbp) of intron 2, a cryptic human exon immediately 3' of this, and a native human exon some 20 kbp downstream. Using two methods, we first carried out the replacement by employing a combination of bacterial artificial chromosome recombineering, classic embryonic stem cell (ESC) targeting, dual selection, and recombinase-driven cassette removal (ESC/Blastocyst Approach). Using a unique second method, we employed the same vector (devoid of its selectable marker cassettes), microinjecting it along with redundant single guide RNAs (sgRNAs) and Cas9 mRNA into mouse zygotes (CRISPR/Zygote Approach). In both instances, we were able to achieve humanization of Bcl2l11 to the extent designed, remove all selection cassettes, and demonstrate the functionality of the conditionally removable, loxP-flanked, 2.9-kbp intronic segment.

Li, S., et al. (2015). "Human Induced Pluripotent Stem Cell NEUROG2 Dual Knockin Reporter Lines Generated by the CRISPR/Cas9 System." Stem Cells Dev **24**(24): 2925-2942.

Human induced pluripotent stem cell (hiPSC) technologies are powerful tools for modeling development and disease, drug screening, and regenerative medicine. Faithful gene targeting in hiPSCs greatly facilitates these applications. We have developed a fast and precise clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9) technology-based method and obtained fluorescent protein and antibiotic resistance dual knockin reporters in hiPSC lines for neurogenin2 (NEUROG2), an important proneural transcription factor. Gene targeting efficiency was greatly improved in CRISPR/Cas9-mediated homology directed recombination (approximately 33% correctly targeted clones) compared to conventional targeting protocol (approximately 3%) at the same locus. No off-target events were detected. In addition, taking the advantage of the versatile applications of the CRISPR/Cas9 system, we designed transactivation components to transiently induce NEUROG2 expression, which helps identify transcription factor binding sites and trans-regulation regions of human NEUROG2. The strategy of using CRISPR/Cas9 genome editing coupled with fluorescence-activated cell sorting of neural progenitor cells in a knockin lineage hiPSC reporter platform might be broadly applicable in other stem cell derivatives and subpopulations.

Ling, K., et al. (2018). "Nanog interaction with the androgen receptor signaling axis induce ovarian cancer stem cell regulation: studies based on the CRISPR/Cas9 system." J Ovarian Res **11**(1): 36.

BACKGROUND: Ovarian cancer stem cells (OCSCs) contribute to the poor prognosis of ovarian cancer. Involvement of the androgen receptor (AR) in the malignant behaviors of other tumors has been reported. However, whether AR associates with Nanog (a stem cell marker) and participates in OCSC functions remain unclear. In this study, we investigated the interaction of Nanog with AR and examined whether this interaction induced stem-like properties in ovarian cancer cells. METHODS: AR and Nanog expression in ovarian tumors was evaluated. Using the CRISPR/Cas9 system, we constructed a Nanog green fluorescent protein (GFP) marker cell model to investigate the expression and co-localization of Nanog and AR. Then, we examined the effect of androgen on the Nanog promoter in ovarian cancer cell lines (A2780 and SKOV3). After androgen or anti-androgen treatment, cell proliferation, migration, sphere formation, colony formation and tumorigenesis were assessed in vitro and in vivo. RESULTS: Both AR and Nanog expression were obviously high in ovarian tumors. Our results showed that Nanog expression was correlated with AR expression. The androgen 5alpha-dihydrotestosterone (DHT) activated Nanog promoter transcription. Meanwhile, Nanog GFP-positive cells treated with DHT exhibited higher levels of proliferation, migration, sphere formation and colony formation. We also observed that the tumorigenesis of Nanog GFP-positive cells was significantly higher than that of the GFP-negative cells. Xenografts of Nanog GFP-positive cells showed significant differences when treated with androgen or anti-androgen drugs in vivo. CONCLUSIONS: The interaction of Nanog with the AR signaling axis might induce or contribute to OCSC regulation. In addition,

androgen might promote stemness characteristics in ovarian cancer cells by activating the Nanog promoter. This finding merits further study because it may provide a new understanding of OCSC regulation from a hormone perspective and lead to the reevaluation of stem cell therapy for ovarian cancer.

Luo, Y., et al. (2016). "Targeted Inhibition of the miR-199a/214 Cluster by CRISPR Interference Augments the Tumor Tropism of Human Induced Pluripotent Stem Cell-Derived Neural Stem Cells under Hypoxic Condition." Stem Cells Int **2016**: 3598542.

The human induced pluripotent stem cell (hiPSC) provides a breakthrough approach that helps overcoming ethical and allergenic challenges posed in application of neural stem cells (NSCs) in targeted cancer gene therapy. However, the tumor-tropic capacity of hiPSC-derived NSCs (hiPS-NSCs) still has much room to improve. Here we attempted to promote the tumor tropism of hiPS-NSCs by manipulating the activity of endogenous miR-199a/214 cluster that is involved in regulation of hypoxia-stimulated cell migration. We first developed a baculovirus-delivered CRISPR interference (CRISPRi) system that sterically blocked the E-box element in the promoter of the miR-199a/214 cluster with an RNA-guided catalytically dead Cas9 (dCas9). We then applied this CRISPRi system to hiPS-NSCs and successfully suppressed the expression of miR-199a-5p, miR-199a-3p, and miR-214 in the microRNA gene cluster. Meanwhile, the expression levels of their targets related to regulation of hypoxia-stimulated cell migration, such as HIF1A, MET, and MAPK1, were upregulated. Further migration assays demonstrated that the targeted inhibition of the miR-199a/214 cluster significantly enhanced the tumor tropism of hiPS-NSCs both in vitro and in vivo. These findings suggest a novel application of CRISPRi in NSC-based tumor-targeted gene therapy.

Martin Gonzalez, J., et al. (2018). "A new genetic tool to improve immune-compromised mouse models: Derivation and CRISPR/Cas9-mediated targeting of NRG embryonic stem cell lines." Genesis **56**(9): e23238.

Development of human hematopoietic stem cells and differentiation of embryonic stem (ES) cells/induced pluripotent stem (iPS) cells to hematopoietic stem cells are poorly understood. NOD (Non-obese diabetic)-derived mouse strains, such as NSG (NOD-Scid-il2Rg) or NRG (NOD-Rag1-il2Rg), are the best available models for studying the function of fetal and adult human hematopoietic cells as well as ES/iPS cell-derived hematopoietic stem cells. Unfortunately, engraftment of human hematopoietic stem cells is very variable in these models. Introduction of additional permissive mutations into these complex genetic backgrounds of the NRG/NSG mice by natural breeding is a very demanding task in terms of time and resources. Specifically, since the genetic elements defining the NSG/NRG phenotypes have not yet been fully characterized, intense backcrossing is required to ensure transmission of the full phenotype. Here we describe the derivation of embryonic stem cell (ESC) lines from NRG pre-implantation embryos generated by in vitro fertilization followed by the CRISPR/CAS9 targeting of the Gata-2 locus. After injection into morula stage embryos, cells from three tested lines gave rise to chimeric adult mice showing high contribution of the ESCs (70%-100%), assessed by coat color. Moreover, these lines have been successfully targeted using Cas9/CRISPR technology, and the mutant cells have been shown to remain germ line competent. Therefore, these new NRG ESC lines combined with genome editing nucleases bring a powerful genetic tool that facilitates the generation of new NOD-based mouse models with the aim to improve the existing xenograft models.

Mosqueira, D., et al. (2018). "CRISPR/Cas9 editing in human pluripotent stem cell-cardiomyocytes highlights arrhythmias, hypocontractility, and energy depletion as potential therapeutic targets for hypertrophic cardiomyopathy." Eur Heart J **39**(43): 3879-3892.

Aims: Sarcomeric gene mutations frequently underlie hypertrophic cardiomyopathy (HCM), a prevalent and complex condition leading to left ventricle thickening and heart dysfunction. We evaluated isogenic genome-edited human pluripotent stem cell-cardiomyocytes (hPSC-CM) for their validity to model, and add clarity to, HCM. Methods and results: CRISPR/Cas9 editing produced 11 variants of the HCM-causing mutation c.C9123T-MYH7 [(p.R453C-beta-myosin heavy chain (MHC)] in 3 independent hPSC lines. Isogenic sets were differentiated to hPSC-CMs for high-throughput, non-subjective molecular and functional assessment using 12 approaches in 2D monolayers and/or 3D engineered heart tissues. Although immature, edited hPSC-CMs exhibited the main hallmarks of HCM (hypertrophy, multi-nucleation, hypertrophic marker expression, sarcomeric disarray). Functional evaluation supported the energy depletion model due to higher metabolic respiration activity, accompanied by abnormalities in calcium handling, arrhythmias, and contraction force. Partial phenotypic rescue was achieved with ranolazine but not omecamtiv mecarbil, while RNAseq highlighted potentially novel molecular targets. Conclusion: Our holistic and comprehensive approach

showed that energy depletion affected core cardiomyocyte functionality. The engineered R453C-betaMHC-mutation triggered compensatory responses in hPSC-CMs, causing increased ATP production and alphaMHC to energy-efficient betaMHC switching. We showed that pharmacological rescue of arrhythmias was possible, while MHY7: MYH6 and mutant: wild-type MYH7 ratios may be diagnostic, and previously undescribed lncRNAs and gene modifiers are suggestive of new mechanisms.

Noack, C., et al. (2017). "Generation of a KLF15 homozygous knockout human embryonic stem cell line using paired CRISPR/Cas9n, and human cardiomyocytes derivation." Stem Cell Res **23**: 127-131.

Krueppel-like factor 15 (KLF15) is abundantly expressed in liver, kidney, and muscle, including myocardium. In the adult heart KLF15 is important to maintain homeostasis and to repress hypertrophic remodeling. We generated a homozygous hESC KLF15 knockout (KO) line using paired CRISPR/Cas9n. KLF15-KO cells maintained full pluripotency and differentiation potential as well as genomic integrity. We demonstrated that KLF15-KO cells can be differentiated into morphologically normal cardiomyocytes turning them into a valuable tool for studying human KLF15-mediated mechanisms resulting in human cardiac dysfunction.

Pan, A., et al. (2014). "Enhancing stem cell survival in an ischemic heart by CRISPR-dCas9-based gene regulation." Med Hypotheses **83**(6): 702-705.

Ischemic heart disease has remained the number one killer around the world for over the past 20 years. While stem cell therapy has become a promising new frontier to repair the damaged heart, limited stem cell survivability post-transplantation has precluded widespread use of this therapy. Strategies to genetically modify stem cells to activate pro-survival and anti-apoptotic and anti-inflammatory pathways, such as Akt and heme oxygenase-1, have been shown to improve the lifespan of transplanted stem cells within the ischemic myocardium, but constitutive overexpression of these pathways at high levels has been shown to have side effects. Therefore, more specific and controlled gene activation would be necessary. Current techniques used for gene regulation include zinc finger and TALE proteins, but there are still disadvantages to each of these methods, such as ease and cost of use. Also, those methods use synthesized promoters to express synthesized cDNA, which lack regulatory elements, including introns and 3' untranslated regions for microRNA mediated post-transcriptional regulation. A new novel technique, the CRISPR/dCas9 system, was recently developed as a simple and efficient method for endogenous gene regulation. With its use of single guide chimeric RNA's (sgRNA's), this system has been shown to provide a high level of specificity and efficiency. When targeting different loci, past studies have found that the CRISPR/dCas9 system can activate gene expression at varying levels. In addition, this system makes use of the genome's endogenous regulatory elements, such as the aforementioned introns and 3' UTR's, which can help provide a safer method of gene activation. If targeted to a gene promoting cellular survival or decreasing cell death, it could potentially improve stem cell longevity in a more efficient and controllable manner. As a result, our hypothesis is to use the CRISPR/dCas9 system to activate expression of an anti-inflammatory and anti-apoptotic gene, such as heme oxygenase-1 (HO-1), to an optimal level to increase transplanted stem cell survival while also mitigating its cytotoxic effects due to lack of internal regulation, thus prolonging its effects within the ischemic myocardium leading to greater therapeutic benefit.

Patmanathan, S. N., et al. (2018). "CRISPR/Cas9 in Stem Cell Research: Current Application and Future Perspective." Curr Stem Cell Res Ther **13**(8): 632-644.

The clustered regularly interspaced short palindromic repeats-associated protein 9 or CRISPR/Cas9 system is one of the hottest topics discussed lately due to its robustness and effectiveness in genome editing. The technology has been widely used in life science research including microbial, plant, animal, and human cell studies. Combined with the pluripotency of stem cells, the technology represents a powerful tool to generate various cell types for disease modeling, drug screening, toxicology, and targeted therapies. Generally, the CRISPR/Cas9 system has been applied in genetic modification of pluripotent or multipotent stem cells, after which the cells are differentiated into specific cell types and used for functional analysis or even clinical transplantation. Recent advancement in CRISPR/Cas9 technology has widened the scope of stem cell research and its therapeutic application. This review provides an overview of the current application and the prospect of CRISPR/Cas9 technology, particularly in stem cell research and therapy.

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.

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.

Sato, M., et al. (2016). "Efficient CRISPR/Cas9-based gene correction in induced pluripotent stem cells established from fibroblasts of patients with sickle cell disease." Stem Cell Investig **3**: 78.

Sato, T., et al. (2015). "Genome Editing in Mouse Spermatogonial Stem Cell Lines Using TALEN and Double-Nicking CRISPR/Cas9." Stem Cell Reports **5**(1): 75-82.

Mouse spermatogonial stem cells (SSCs) can be cultured for multiplication and maintained for long periods while preserving their spermatogenic ability. Although the cultured SSCs, named germline stem (GS) cells, are targets of genome modification, this process remains technically difficult. In the present study, we tested TALEN and double-nicking CRISPR/Cas9 on GS cells, targeting Rosa26 and Stra8 loci as representative genes dispensable and indispensable in spermatogenesis, respectively. Harvested GS cell colonies showed a high targeting efficiency with both TALEN and CRISPR/Cas9. The Rosa26-targeted GS cells differentiated into fertility-competent sperm following transplantation. On the other hand, Stra8-targeted GS cells showed defective spermatogenesis following transplantation, confirming its prime role in the initiation of meiosis. TALEN and CRISPR/Cas9, when applied in GS cells, will be valuable tools in the study of spermatogenesis and for revealing the genetic mechanism of spermatogenic failure.

Schwank, G., et al. (2013). "Functional repair of CFTR by CRISPR/Cas9 in intestinal stem cell organoids of cystic fibrosis patients." Cell Stem Cell **13**(6): 653-658.

Single murine and human intestinal stem cells can be expanded in culture over long time periods as genetically and phenotypically stable epithelial organoids. Increased cAMP levels induce rapid swelling of such organoids by opening the cystic fibrosis transmembrane conductor receptor (CFTR). This response is lost in organoids derived from cystic fibrosis (CF) patients. Here we use the CRISPR/Cas9 genome editing system to correct the CFTR locus by homologous recombination in cultured intestinal stem cells of CF patients. The corrected allele is expressed and fully functional as measured in clonally expanded organoids. This study provides proof of concept for gene correction by homologous recombination in primary adult stem cells derived from patients with a single-gene hereditary defect.

Shen, Y., et al. (2018). "Generation of PTEN knockout bone marrow mesenchymal stem cell lines

by CRISPR/Cas9-mediated genome editing." Cytotechnology **70**(2): 783-791.

The tumor suppressor PTEN is involved in the regulation of cell proliferation, lineage determination, motility, adhesion and apoptosis. Loss of PTEN in the bone mesenchymal stem cells (BMSCs) was shown to change their function in the repair tissue. So far, the CRISPR/Cas9 system has been proven extremely simple and flexible. Using this system to manipulate PTEN gene editing could produce the PTEN-Knocking-out (PTEN-KO) strain. We knocked out PTEN in MSCs and validated the expression by PCR and Western blot. To clarify the changes in proliferation, CCK-8 assay was applied. In support, living cell proportion was assessed by Trypan blue staining. For osteogenic and adipogenic induction, cells were cultured in different media for 2 weeks. Oil red staining and alizarin red staining were performed for assessment of osteogenic or adipogenic differentiation. The expression of Id4, Runx2, ALP and PPARgamma was examined by qPCR and immunocytochemistry staining. The PTEN-KO strain was identified by sequencing. The PTEN-KO cells had an increased cell viability and higher survival compared with the wild type. However, decreased expression of Runx2 and PPARgamma was found in the PTEN loss strain after induction, and consistently decreased osteogenic or adipogenic differentiation was observed by alizarin and oil red staining. Together, PTEN-KO strain showed an increased proliferation capability but decreased multi-directional differentiation potential. When BMSCs serve as seed cells for tissue engineering, the PTEN gene may be used as an indicator.

Shetty, D. K. and M. S. Inamdar (2016). "Generation of a heterozygous knockout human embryonic stem cell line for the OCIAD1 locus using CRISPR/CAS9 mediated targeting: BJNhem20-OCIAD1-CRISPR-20." Stem Cell Res **16**(2): 207-209.

Ovarian carcinoma immuno-reactive antigen domain containing 1(OCIAD1) single copy was knocked out generating an OCIAD1 heterozygous knockout human embryonic stem line named BJNhem20-OCIAD1-CRISPR-20. The line was generated using CRISPR-Cas9D10A double nickase knockout strategy (Mali et al., 2013).

Shetty, D. K. and M. S. Inamdar (2016). "Generation of a heterozygous knockout human embryonic stem cell line for the OCIAD1 locus using CRISPR/CAS9 mediated targeting: BJNhem20-OCIAD1-CRISPR-39." Stem Cell Res **16**(2): 308-310.

Ovarian carcinoma immuno-reactive antigen domain containing 1 (OCIAD1) single copy was knocked out generating an OCIAD1 heterozygous knockout human embryonic stem line named BJNhem20-OCIAD1-CRISPR-39. The line was generated using CRISPR-Cas9D10A double nickase knockout strategy (Mali et al., 2013).

Sweeney, C. L., et al. (2017). "CRISPR-Mediated Knockout of Cybb in NSG Mice Establishes a Model of Chronic Granulomatous Disease for Human Stem-Cell Gene Therapy Transplants." Hum Gene Ther **28**(7): 565-575.

Chronic granulomatous disease (CGD) is characterized by defects in the production of microbicidal reactive oxygen species (ROS) by phagocytes. Testing of gene and cell therapies for the treatment of CGD in human hematopoietic cells requires preclinical transplant models. The use of the lymphocyte-deficient NOD.Cg-Prkdc (scid) Il2rg (tm1Wjl/)SzJ (NSG) mouse strain for human hematopoietic cell xenografts to test CGD therapies is complicated by the presence of functional mouse granulocytes capable of producing ROS for subsequent bacterial and fungal killing. To establish a phagocyte-defective mouse model of X-linked CGD (X-CGD) in NSG mice, clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 was utilized for targeted knockout of mouse Cybb on the X-chromosome by microinjection of NSG mouse zygotes with Cas9 mRNA and CRISPR single-guide RNA targeting Cybb exon 1 or exon 3. This resulted in a high incidence of indel formation at the CRISPR target site, with all mice exhibiting deletions in at least one Cybb allele based on sequence analysis of tail snip DNA. A female mouse heterozygous for a 235-bp deletion in Cybb exon 1 was bred to an NSG male to establish the X-CGD NSG mouse strain, NSG.Cybb [KO]. Resulting male offspring with the 235 bp deletion were found to be defective for production of ROS by neutrophils and other phagocytes, and demonstrated increased susceptibility to spontaneous bacterial and fungal infections with granulomatous inflammation. The establishment of the phagocyte-defective NSG.Cybb [KO] mouse model enables the in vivo assessment of gene and cell therapy strategies for treating CGD in human hematopoietic cell transplants without obfuscation by functional mouse phagocytes, and may also be useful for modeling other phagocyte disorders in humanized NSG mouse xenografts.

Tang, C. C., et al. (2017). "Generation of a Bag1 homozygous knockout mouse embryonic stem cell line using CRISPR/Cas9." Stem Cell Res **21**: 29-31.

Bag1 transcribes a multifunctional protein that participates in many important biological processes such as cell apoptosis, proliferation, differentiation and embryo development. Despite numerous published studies, the role of Bag1 in the context of embryonic

stem (ES) cells, has not been explored. To investigate the function of Bag1 in ES cells, we generated mutant Bag1(-/-) ES cells using the CRISPR/Cas9 system. We established that the Bag1 double knockout ES cell line maintained their pluripotency, possessed a normal karyotype and the ability to differentiate into all three germ layers.

Veres, A., et al. (2014). "Low incidence of off-target mutations in individual CRISPR-Cas9 and TALEN targeted human stem cell clones detected by whole-genome sequencing." Cell Stem Cell **15**(1): 27-30.

Genome editing has attracted wide interest for the generation of cellular models of disease using human pluripotent stem cells and other cell types. CRISPR-Cas systems and TALENs can target desired genomic sites with high efficiency in human cells, but recent publications have led to concern about the extent to which these tools may cause off-target mutagenic effects that could potentially confound disease-modeling studies. Using CRISPR-Cas9 and TALEN targeted human pluripotent stem cell clones, we performed whole-genome sequencing at high coverage in order to assess the degree of mutagenesis across the entire genome. In both types of clones, we found that off-target mutations attributable to the nucleases were very rare. From this analysis, we suggest that, although some cell types may be at risk for off-target mutations, the incidence of such effects in human pluripotent stem cells may be sufficiently low and thus not a significant concern for disease modeling and other applications.

Wei, H., et al. (2018). "CRISPR/Cas9 Gene editing of RyR2 in human stem cell-derived cardiomyocytes provides a novel approach in investigating dysfunctional Ca (2+) signaling." Cell Calcium **73**: 104-111.

Type-2 ryanodine receptors (RyR2s) play a pivotal role in cardiac excitation-contraction coupling by releasing Ca (2+) from sarcoplasmic reticulum (SR) via a Ca (2+) -induced Ca (2+) release (CICR) mechanism. Two strategies have been used to study the structure-function characteristics of RyR2 and its disease associated mutations: (1) heterologous cell expression of the recombinant mutant RyR2s, and (2) knock-in mouse models harboring RyR2 point mutations. Here, we establish an alternative approach where Ca (2+) signaling aberrancy caused by the RyR2 mutation is studied in human cardiomyocytes with robust CICR mechanism. Specifically, we introduce point mutations in wild-type RYR2 of human induced pluripotent stem cells (hiPSCs) by CRISPR/Cas9 gene editing, and then differentiate them into cardiomyocytes. To verify the reliability of this approach, we introduced the same disease-associated RyR2 mutation, F2483I, which was studied by us in hiPSC-derived cardiomyocytes (hiPSC-CMs) from a patient biopsy. The gene-edited F2483I hiPSC-CMs exhibited longer and wandering Ca (2+) sparks, elevated diastolic Ca (2+) leaks, and smaller SR Ca (2+) stores, like those of patient-derived cells. Our CRISPR/Cas9 gene editing approach validated the feasibility of creating myocytes expressing the various RyR2 mutants, making comparative mechanistic analysis and pharmacotherapeutic approaches for RyR2 pathologies possible.

Wei, R., et al. (2018). "Construction of a GLI3 compound heterozygous knockout human embryonic stem cell line WAe001-A-20 by CRISPR/Cas9 editing." Stem Cell Res **32**: 139-144.

The human GLI3 protein has a dual function as a transcriptional activator or repressor of hedgehog signaling, depending on the proteolytic processing forms of GLI3. In this study, we established a compound heterozygous GLI3 mutant human embryonic stem cell line (WAe001-A-20) through CRISPR/Cas9 editing. The WAe001-A-20 cells carried two deletions on two different alleles of exon 2 of GLI3, respectively, which resulted in a frame shift and early termination in the translation of GLI3. Moreover, WAe001-A-20 maintains a normal karyotype, parental cell morphology, pluripotent phenotype and the ability to differentiate into three germ layers. Resource table.

Wettstein, R., et al. (2016). "Generation of a Knockout Mouse Embryonic Stem Cell Line Using a Paired CRISPR/Cas9 Genome Engineering Tool." Methods Mol Biol **1341**: 321-343.

CRISPR/Cas9, originally discovered as a bacterial immune system, has recently been engineered into the latest tool to successfully introduce site-specific mutations in a variety of different organisms. Composed only of the Cas9 protein as well as one engineered guide RNA for its functionality, this system is much less complex in its setup and easier to handle than other guided nucleases such as Zinc-finger nucleases or TALENs.Here, we describe the simultaneous transfection of two paired CRISPR sgRNAs-Cas9 plasmids, in mouse embryonic stem cells (mESCs), resulting in the knockout of the selected target gene. Together with a four primer-evaluation system, it poses an efficient way to generate new independent knockout mouse embryonic stem cell lines.

Wu, F., et al. (2018). "Generation of a SMO homozygous knockout human embryonic stem cell

line WAe001-A-16 by CRISPR/Cas9 editing." Stem Cell Res **27**: 5-9.

The human SMO protein encoded by the smoothened (SMO) gene acts as a positive mediator for Hedgehog signaling. This pathway regulates many cellular activities, developmental morphogenesis, and tumorigenesis. Using CRISPR/Cas9 to edit human embryonic stem cell line WA01 (H1), we established a SMO mutant cell line (WAe001-A-16). This cell line has a 40bp homozygous deletion in exon 2 of SMO leading to a shift in the open reading frame and early termination at amino acid position 287. WAe001-A-16 maintains a normal karyotype, parental cell morphology, pluripotency markers, and the capacity to differentiate into all three germline layers.

Xu, G., et al. (2018). "Generation of a GDE heterozygous mutation human embryonic stem cell line WAe001-A-14 by CRISPR/Cas9 editing." Stem Cell Res **27**: 38-41.

Glycogen debranching enzyme (GDE) plays a critical role in glycogenolysis. Mutations in the GDE gene are associated with a metabolic disease known as glycogen storage disease type III (GSDIII). We generated a mutant GDE human embryonic stem cell line, WAe001-A-14, using the CRISPR/Cas9 editing system. This cell line contains a 24-nucleotide deletion within exon-13 of GDE, resulting in 8 amino acids (TRLGISSL) missing of the GDE protein from amino acid position 567 to 575. The WAe001-A-14 cell line maintains typical stem cell morphology, pluripotency and in vitro differentiation potential, and a normal karyotype.

Xu, Y., et al. (2017). "Generation of an ASGR1 homozygous mutant human embryonic stem cell line WAe001-A-6 using CRISPR/Cas9." Stem Cell Res **22**: 29-32.

The gene asialoglycoprotein receptor 1 (ASGR1) encodes a subunit of the asialoglycoprotein receptor. Here we report the generation of a human embryonic stem cell line WAe001-A-6 harbouring homozygous ASGR1 mutations using CRISPR/Cas9. The mutation involves a 37bp deletion, resulting in a frame shift. The homozygous knockout WA01 cell line maintains a normal karyotype, typical stem cell morphology, pluripotency and differentiation potential in vitro.

Xue, Y., et al. (2017). "Establishment of a congenital tooth agenesis related gene MSX1 knockout human embryonic stem cell lines by CRISPR-Cas9 technology." Stem Cell Res **24**: 151-154.

Human MSX1 gene is mapped to chromosome 4 and encodes a 303aa homeobox protein MSX1. MSX1 expression appears during early tooth development of vertebrate embryogenesis. Mutations in this protein are related to human tooth anomalie, cleft lip and palate and congenital ectodermal dysplasia syndrome. Most of the confirmed pathogenic mutations are located in exon2 encoded homeobox domain. Here, we report the establishment of MSX1 gene knockout human embryonic stem (hES) cell lines by CRISPR-Cas9 technology. These cell lines provide good materials for further studies of the roles MSX1 plays in human tooth development and congenital tooth agenesis.

Yuan, F., et al. (2017). "Generation of a KCNJ11 homozygous knockout human embryonic stem cell line WAe001-A-12 using CRISPR/Cas9." Stem Cell Res **24**: 89-93.

The ATP-sensitive potassium channel is an octameric complex, and one of its subunits, namely Kir6.2, is encoded by the KCNJ11 gene. Mutations in KCNJ11 result in hyperinsulinism or diabetes mellitus, associated with abnormal insulin secretion. Here, using CRISPR/Cas9 editing, we established a homozygous mutant KCNJ11 cell line, WAe001-A-12, which was generated by a 62-bp deletion in the coding sequence of the human embryonic stem cell line H1. It was confirmed that this deletion in the KCNJ11 gene did not affect the protein expression levels of key pluripotent factors. Additionally, normal karyotype and differentiation potency were observed for the cell line.

Yuan, F., et al. (2018). "Generation of an ASS1 heterozygous knockout human embryonic stem cell line, WAe001-A-13, using CRISPR/Cas9." Stem Cell Res **26**: 67-71.

The ASS1 gene encodes argininosuccinate synthetase-1, a cytosolic enzyme with a critical role in the urea cycle. Mutations are found in all ASS1 exons and cause the autosomal recessive disorder citrullinemia. Using CRISPR/Cas9-editing, we established the WAe001-A-13 cell line, which was heterozygous for an ASS1 mutation, from the human embryonic stem cell line H1. The WAe001-A-13 cell line maintained the pluripotent phenotype, the ability to differentiate into all three germ layers and a normal karyotype.

Zhang, Y., et al. (2016). "Generation of a human induced pluripotent stem cell line via CRISPR-Cas9 mediated integration of a site-specific heterozygous mutation in CHMP2B." Stem Cell Res **17**(1): 148-150.

Frontotemporal dementia (FTD) is an early onset neurodegenerative disease. Mutations in several genes cause familial FTD and one of them is charged multivesicular body protein 2B (CHMP2B) on chromosome 3 (FTD3), a component of the endosomal sorting complex required for transport III (ESCRT-III).

We have generated an induced pluripotent stem cell (iPSC) line of a healthy individual and inserted the CHMP2B IVS5AS G-C gene mutation into one of the alleles, resulting in aberrant splicing. This human iPSC line provides an ideal model to study CHMP2B-dependent phenotypes of FTD3.

Zhang, Y., et al. (2016). "Generation of a human induced pluripotent stem cell line via CRISPR-Cas9 mediated integration of a site-specific homozygous mutation in CHMP2B." Stem Cell Res **17**(1): 151-153.

Frontotemporal dementia (FTD) is an early onset neurodegenerative disease. Mutations in several genes cause familial FTD and one of them is charged multivesicular body protein 2B (CHMP2B) on chromosome 3 (FTD3), a component of the endosomal sorting complex required for transport III (ESCRT-III). We have generated an induced pluripotent stem cell (iPSC) line of a healthy individual and inserted the CHMP2B IVS5AS G-C gene mutation into both alleles, resulting in aberrant splicing. This human iPSC line provides an ideal model to study CHMP2B-dependent phenotypes of FTD3.

Zhou, J., et al. (2016). "Generation of Human Embryonic Stem Cell Line Expressing zsGreen in Cholinergic Neurons Using CRISPR/Cas9 System." Neurochem Res **41**(8): 2065-2074.

Lineage specific human embryonic stem cell (hESC) reporter cell line is a versatile tool for biological studies on real time monitoring of differentiation, physiological and biochemical features of special cell types and pathological mechanism of disease. Here we report the generation of ChAT-zsGreen reporter hESC line that express zsGreen under the control of the choline acetyltransferase (ChAT) promoter using CRISPR (Clustered Regularly Interspersed Short Palindromic Repeats)/Cas9 system. We show that the ChAT-zsGreen hESC reporter cell lines retain the features of undifferentiated hESC. After cholinergic neuronal differentiation, cholinergic neurons were clearly labeled with green fluorescence protein (zsGreen). The ChAT-zsGreen reporter hESC lines are invaluable not only for the monitoring cholinergic neuronal differentiation but also for study physiological and biochemical hallmarks of cholinergic neurons.

Zuo, Q., et al. (2017). "CRISPR/Cas9-Mediated Deletion of C1EIS Inhibits Chicken Embryonic Stem Cell Differentiation Into Male Germ Cells (Gallus gallus)." J Cell Biochem **118**(8): 2380-2386.

We previously found that C1EIS is preferentially expressed in Chicken spermatogonial stem cells (SSCs) by RNA sequencing (RNA-seq), so our current study focused on C1EIS's role in Chicken embryonic stem cells (ESCs) differentiation into male germ cells. We constructed a CRISPR/Cas9 vector targeting C1EIS. T7 endonuclease I (T7EI) digestion method and sequencing of TA cloning were used to detect the knock-out efficiency of the Single guide RNA (sgRNA) after the cas9/gRNA vector transfected into D fibroblasts 1(DF-1), ESCs, and Chicken embryos. The results showed that CRISPR/Cas9 gene knockout efficiency is about 40%. Differentiation of the targeted ESCs into SSCs was inhibited at the embryoid body stage due to C1EIS deficiency. Immunofluorescent staining revealed that the mutagenized ESCs (RA (Retinoic Acid) with C1EIS Knock out) expressed lower levels of integrin alpha6 and integrin beta1 compared to wild type cells. Quantitative real-time PCR (QRT-PCR) revealed Oct4 and Sox2 expression significantly increased, contrarily integrin beta1 and Stra8 expression significantly decreased than RA induced group and RA with C1EIS Overexpression. During retinoic acid-induced differentiation, knockout of C1EIS in ESCs inhibited formation of SSC-like cells, suggesting C1EIS plays a vital role in promoting differentiation of avian ESCs to SSCs by regulating expression of multiple pluripotency-related genes. J. Cell. Biochem. 118: 2380-2386, 2017. (c) 2017 Wiley Periodicals, Inc.

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