Human Genetics (2019) 138:1217–1225
https://doi.org/10.1007/s00439-019-02071-z

REVIEW

CRISPR/Cas9 facilitates genomic editing for large-scale functional studies in pluripotent stem cell cultures

Xiao-Fei Li[1] · Yong-Wei Zhou[1] · Peng-Fei Cai[1] · Wei-Cong Fu[1] · Jin-Hua Wang[1] · Jin-Yang Chen[2] · Qi-Ning Yang[1]

Received: 8 July 2019 / Accepted: 4 October 2019 / Published online: 12 October 2019

Abstract

Pluripotent stem cell (PSC) cultures form an integral part of biomedical and medical research due to their capacity to rapidly proliferate and differentiate into hundreds of highly specialized cell types. This makes them a highly useful tool in exploring human physiology and disease. Genomic editing of PSC cultures is an essential method of attaining answers to basic physiological functions, developing in vitro models of human disease, and exploring potential therapeutic strategies and the identification of drug targets. Achieving reliable and efficient genomic editing is an important aspect of using large-scale PSC cultures. The CRISPR/Cas9 genomic editing tool has facilitated highly efficient gene knockout, gene correction, or gene modifications through the design and use of single-guide RNAs which are delivered to the target DNA via Cas9. CRISPR/Cas9 modification of PSCs has furthered the understanding of basic physiology and has been utilized to develop in vitro disease models, to test therapeutic strategies, and to facilitate regenerative or tissue repair approaches. In this review, we discuss the benefits of the CRISPR/Cas9 system in large-scale PSC cultures.

Introduction

Stem cells can be found in embryonic organisms, such as humans and other mammals. These cells have the capacity to differentiate into more than 200 different and specialized cell types (Reubinoff et al. 2000; Takahashi and Yamanaka 2006). In vivo, stem cells are utilized by the organism to repair damage and to maintain homeostasis (Zhang et al. 2017). Furthermore, these cells can be isolated and pluripotent stem cell (PSC) cultures can be generated (McKee and Chaudhry 2017). Cultures of human pluripotent stem cells (hPSCs) can be generated from: (1) the controversial human embryonic stem cells (hESCs), which are derived from preimplantation blastocysts; and (2) the rather less controversial induced pluripotent stem cells (hiPSCs) that are derived from adult sources, such as a tissue sample collected via biopsy and genetically induced into an embryonic-like state (Thomson et al. 1998; Takahashi and Yamanaka 2006). Cultures of PSCs can also be generated from other sources, such as rodents or fish; however, human-derived PSC cultures are the most beneficial for understanding human physiology and disease. Once a line of cultured pluripotent stem cells has been established, these cells can be maintained in culture for a very long or an indefinite period (McKee and Chaudhry 2017). These PSCs can readily proliferate and easily accommodate the generation of large-scale cultures, without losing the morphological or physiological integrity of the cells (Sterneckert et al. 2014).

The large-scale culture of hPSCs has become a popular and valuable tool in medical and biomedical research. PSC cultures have been widely utilized to answer questions related to normal physiology, such as exploring signaling and trafficking pathways, regulatory mechanisms, and normal homeostatic functions (Chen et al. 2015; Zhang et al. 2017). Through genomic editing, these PSC cell lines facilitate: (1) the development of in vitro disease models, which allows the large-scale study of disease development, progression, and disease-specific mutations (Daley 2012; Chen et al. 2014; Liu and Deng 2016); (2) drug screening, identification of potential drug targets, and other pharmacological interventions (Engle and Puppala 2013; Mohamed et al. 2019); (3) the development of regenerative medicine and other cell-based therapies (Mandal et al. 2014; Liu and Deng

✉ Qi-Ning Yang
jhyangqn@sina.com

[1] Department of Joint Surgery, Jinhua Municipal Central Hospital, No. 365 Renmin East Road, Wucheng District, Jinhua 321000, Zhejiang, People's Republic of China

[2] Research and Development Department, Zhejiang Healthfuture Institute for Cell-Based Applied Technology, Hangzhou 310052, Zhejiang, People's Republic of China

2016); and (4) the enhanced understanding of the physiological roles of specific proteins in cell function (Mandegar et al. 2016; Wang et al. 2017).

As PSC culture has become more accessible and integral to medical research, tools were developed to facilitate more efficient genomic editing, encompassing gene knockout, gene knock-in, and controlled gene expression. Genomic editing tools possess the potential to aid the treatment of diseases with a genomic basis, such as cystic fibrosis and diabetes (Ding et al. 2013a; Ormond et al. 2017). The two categories of gene therapies include germline therapy (targets reproductive cells) and somatic therapy (targets non-reproductive cells). Germline therapy could prevent the inheritance of diseases, while somatic therapy could slow or reverse the disease processes. Genomic editing tools have included zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), RNA interference (RNAi), and, most recently, clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) also known as CRISPR/Cas9 (Cox et al. 2015). Each of these tools has enabled significant progress in medical research, but CRISPR, in particular, has been integral in expanding the scope of genomic modifications in PSC research with its ease of facilitating the generation of large-scale studies (Doudna and Charpentier 2014; Ding et al. 2013b). This review will explore how CRISPR/Cas genomic modification in large-scale PSC cultures has facilitated the progress of in-depth functional studies with respect to disease pathogenesis, drug targeting, and other therapeutic strategies.

CRISPR/Cas: an overview

Since its introduction to the scientific community, CRISPR/Cas has become an integral part of many facets of scientific research, revolutionizing both in vitro and in vivo genomic editing. CRISPR was originally identified in bacteria and archaea, and forms part of the prokaryotic adaptive immune system, where some have described its function as similar to that of RNA interference (Makarova et al. 2006; Barrangou 2015a). The CRISPR immune system targets and genomically modifies foreign species, such as viruses, offering the host organism protection (Barrangou and Doudna 2016). The process of how the CRISPR immune system functions has been widely described (Jinek et al. 2013; Barrangou 2015b; Rath et al. 2015; Hryhorowicz et al. 2017); CRISPR facilitates the uptake of foreign DNA which results in the formation of CRISPR-RNA (crRNA). This crRNA then forms a guide RNA (gRNA), which further guides a recruited CRISPR-associated protein (Cas) towards the target foreign DNA sequence. Following the identification of the complementary sequence, the Cas endonuclease introduces double-strand breaks into the target DNA sequence, enabling its cleavage (Garneau et al. 2010; Gasiunas et al. 2012; Brouns et al. 2018). Of these systems, the type II CRISPR/Cas9 system found in *Streptococcus pyogenes* is perhaps the best known. Through adaptation, it gave rise to the now widely used CRISPR/Cas9 genome editing system, which channeled the gene editing efficacy of the prokaryotic CRISPR system for use in eukaryotic genomic editing (Mojica et al. 2009; Mojica and Montoliu 2016; Tadić et al. 2019).

Through simplification of the original type II system, the CRISPR/Cas9 technology facilitates genomic editing via a single-guide RNA (gRNA) which can be adjusted to target any desired DNA locus (Makarova et al. 2011; Barrangou 2015b) (Fig. 1). This gRNA guides the Cas9 endonuclease to the target sequence, which is identified by the protospacer adjacent motif (PAM). The PAM is recognized by a conserved sequence flanking the target sequence (Mojica et al. 2009; Vercoe et al. 2013; Anders et al. 2014). Once bound to the PAM, Cas 9 searches for the complementary DNA, which is identified by the first 10–12 base pairs of the gRNA (Anders et al. 2014; Barrangou and Doudna 2016). The Cas9 and gRNA then insert double-strand breaks into the DNA to cleave or insert DNA sequence at the target location (Garneau et al. 2010; Anderson et al. 2015).

For many biomedical/medical researchers, including those working with PSCs, one of the major virtues of the CRISPR/Cas9 system is its ease of use. The CRISPR/Cas9 process is dependent on DNA and RNA recognition, so, for each new desired target DNA loci, only the gRNA needs to be redesigned. This process has been made considerably easier by the emergence of user-friendly gRNA design tools (Brazelton et al. 2015; Mohr et al. 2016; Cao et al. 2016). With a well-designed gRNA, the CRISPR/Cas9 tool has been shown to facilitate low levels of off-target specificity and relatively high editing specificity (Mali et al. 2013; Anderson et al. 2015; Hendel et al. 2015). Within PSC-based research, CRISPR/Cas9 is being widely utilized to enable genomic modifications, such as gene knock-in, gene knockout, and mutation of DNA in large-scale cultures, facilitating key areas of PSC research interests like basic physiology, disease modeling, and drug screening.

CRISPR/Cas9 predecessors

The need to facilitate genomic editing has been an essential aspect of biomedical and medical research since well before the inception of the CRISPR/Cas9 system. The desired traits of a genomic editing system are high target specificity, ease of use, and efficiency. When utilized in a PSC culture, the genetically modified PSCs must be readily incorporated into large-scale cultures, to facilitate studies exploring specific

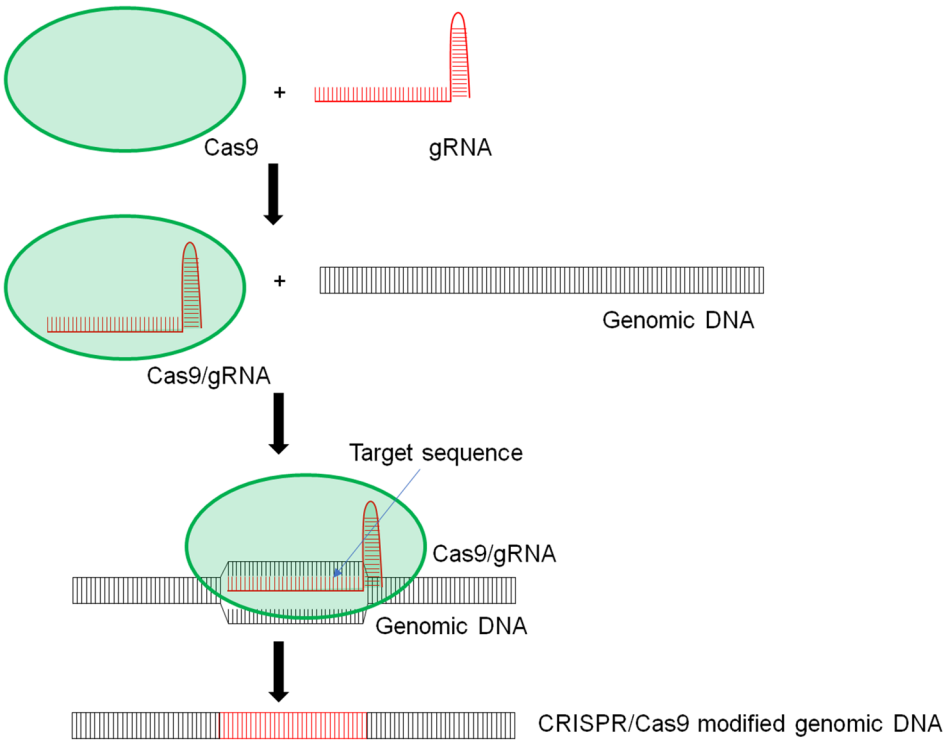

Fig. 1 A simplified schematic of the CRISPR/Cas9 genomic editing system. Guide RNA (gRNA) guides a recruited Cas9 endonuclease to the target DNA sequence in genomic DNA. Cas9 then introduces double-strand breaks into the target DNA sequence, facilitating binding of the gRNA. Once bound, the gRNA either cleaves or modifies the target DNA sequence

protein function, creating disease models and identification of potential drug targets, among others. To accommodate these needs, various genomic editing methods have been utilized in PSC-based research. The most prominent of these methods include ZFNs and TALENs.

Zinc finger nucleases (ZFNs)

One of the earliest wide-spread genomic editing tools was ZFNs, which were first demonstrated in 1996. Commercially available ZFNs are essentially restriction enzymes which were designed to target DNA sequences of interest. This genomic editing tool is comprised of a zinc finger DNA-binding domain fused with a DNA-cleavage domain (Kim et al. 1996; Carroll 2011). Similar to the CRISPR/Cas9 system, the ZFN system is derived from a naturally occurring source. In the case of ZFNs, this is the natural type IIS restriction enzyme FOKI (Li et al. 1992; Kim and Chandrasegaran 1994). Similar to CRISPR/Cas9, ZFN-mediated genomic editing recognizes a predetermined DNA sequence. However, unlike CRISPR/Cas9, ZFNs target a sequence of DNA triplets. As each ZFN can recognize 3-6 nucleotide triplets, most ZFN systems employ two or more zinc fingers to improve target DNA recognition and to reduce off-target activity (Smith 2000; Miller et al. 2007; Maeder et al. 2008; Doyon et al. 2010). Upon binding to their target sequence, ZFNs cleave the target DNA and lead to genomic modification as a result of endogenous DNA repair (Urnov et al. 2010; Carroll 2011). Unlike CRISPR/Cas9, the generation of ZFNs to modify target DNA is not quite as simple as designing a new gRNA. Furthermore, ZFNs do not have as high a degree of accuracy as the CRISPR/Cas9 system (Cornu et al. 2008; Ramirez et al. 2008). However, ZFNs have widely and successfully been utilized in the genomic editing of PSCs for disease modeling and for therapeutic purposes, and are still a popular and reliable method for genomic modifications (Merkert and Martin 2016; Gutierrez-Guerrero et al. 2018; Huang et al. 2018).

Transcription activator-like effector nucleases (TALENs)

TALENs were the next breakthrough in genomic editing, following ZFNs, and the two tools apply some of the same approaches. Genome editing technique using TALENs was first published in 2010 (Christian et al. 2010). Both TALENs and ZFNs are dependent upon a mechanism of protein-DNA recognition, whereas CRISPR/Cas9 utilizes a system of RNA–DNA recognition (Joung and Sander 2013). Like ZFNs, TALENs are engineered restriction enzymes that incorporate a DNA-binding domain and a DNA-cleavage domain. They differ primarily in that ZFNs employ a zinc finger-fused DNA-binding domain, whereas TALENs use a transcription activator-like effector (TALE) DNA-binding domain (Joung and Sander 2013). TALENs can be engineered to target any DNA sequence of interest and following transfection into the cell (including PSCs), TALENs like CRISPR/Cas9 introduce double-strand breaks into the target

DNA sequence, and the modified sequence introduced as a result of endogenous DNA repair. TALENs are considered to be more user-friendly than ZFNs and have a greater degree of specificity, while still being less effective than CRISPR/Cas9 (Hockemeyer et al. 2011; Reyon et al. 2013; Ding et al. 2013a, b). Like ZFNs and CRISPR/Cas9, TALENs remain a powerful tool in genomic editing and have successfully been utilized in PSC culture (Suzuki et al. 2016; Nii et al. 2016; Nakano et al. 2019).

CRISPR/Cas9: functional use in large-scale PSC cultures

The ability to accurately and reliably model disease is a fundamental cornerstone of biomedical and medical research. Disease modeling is essential to effectively identify disease-specific mutations, to explore disease-related physiology and pathogenesis as well as to ultimately develop therapeutic strategies. The most commonly used methods to model disease include transgenic animal models, often rodents such as rats and mice, or various cell lines. While each of these modeling approaches has aided the understanding of human disease, they are inherently flawed. In the case of cell line-based disease modeling, the cell lines are useful in providing insights into specific proteins, receptors, mutations, or overall changes with respect to cell survival and general function, protein signaling, and trafficking (Sterneckert et al. 2014; Trounson and McDonald 2015; Zhang et al. 2017). However, these cell lines are often not physiologically relevant in terms of source or cell type, meaning that the effects observed do not reliably translate to human disease. Furthermore, they often fail to effectively reproduce the disease microenvironment or genotype; hence, they lose the overall complexity of the disease being studied (Sterneckert et al. 2014).

Transgenic animal models enable an in vivo or organism-wide study of disease. They facilitate the replication of an organism-wide phenotype and microenvironment, and enable the study of disease pathogenesis and phenotype. However, these transgenic animal models are severely limited in their scope. For many diseases, the precise cause is unknown, so these models are generated based on limited information mimicking disease-like traits rather than the condition itself. In other models, the mutations expressed are typically limited to familial disease variants, even when the disease of interest is primarily sporadic in humans (Vandamme 2014). Additionally, the physiology of transgenic models is usually significantly different than that of a human, drawing into question the reliability of these models (Vandamme 2014).

The inefficiency of these transgenic animal disease models is particularly obvious when looking at clinical trial outcomes. Many of the treatments entering clinical trials show remarkable promise in in vivo disease models, often ameliorating or eliminating disease-related pathology. However, most of these treatments fail their clinical trials (Wong et al. 2019). This significant failure highlights the need for a more reliable and physiologically relevant means of modeling human diseases. Ideologically, the optimal way to understand human disease is, of course, to study it in humans. However, the capacity for conducting human in vivo studies is extremely limited due to ethical considerations. While human data can be obtained, this is often from non-invasive measures, or collection of treatment-related samples, which is incredibly valuable. Access to primary cell cultures generated from patient samples is also of use; however, these samples are often limited in terms of availability and sample size (Sterneckert et al. 2014). This limited sample availability places limitations on the scope of the research that can be performed. To facilitate the large-scale research needed to explore and treat human disease, PSCs have become an integral tool in disease modeling.

PSC-derived disease models have been generated for a wide range of diseases, including neurological, cancers, and hepatic and cardiac diseases, among others. These disease models can be generated using either PSCs derived from healthy sources such as embryonic stem cells (ESCs) or healthy adult sources, or they can be collected from the patients themselves, to readily access a specific disease phenotype. The CRISPR/Cas9 system has garnered wide-spread popularity both in the creation of isogenic disease models using healthy PSC sources, as well as in the testing and identification of potential therapeutic strategies within patient-derived PSC cultures. Some of the studies utilizing CRISPR/Cas9 in PSC-based research are summarized in Table 1.

Generation of PSC-derived isogenic disease models

The capacity to rapidly proliferate yielding large-scale cultures and their ability to differentiate into many cell types makes PSCs ideal tools for modeling human disease. As the popularity of the CRISPR/Cas9 genomic editing system grew, the availability of tools to more efficiently design gRNAs also expanded (Brazelton et al. 2015; Mohr et al. 2016; Cao et al. 2016). Through these tools, gRNAs can be more rapidly designed and generated. With the ability to generate more accurate gRNAs, CRISPR/Cas9 has been found to modify target gene sequences with approximately 80% target efficiency (Ding et al. 2013b). CRISPR/Cas9-modified, PSC-derived isogenic disease models can be produced in as little as 2 weeks, and of those that have been developed, CRISPR/Cas9 and other related CRISPR technologies have been used to effectively knockout sequences, knock-in DNA, and to insert a variety of mutations, such as point or missense mutations to disrupt normal processes

Table 1 Examples of studies utilizing CRISPR/Cas9 in pluripotent stem cell (PSC)-based research

Cell type	Study type/condition	CRISPR/modification	Outcomes	References
ESC	Basic biology	Inactivation/Depletion of *Mettl3*	Impaired differentiation	Batista et al. (2014)
hESC, iPSC	Basic biology	Knock-in of FRT sequences	Develop inducible gene knockout model	Chen et al. (2015)
ESC	Basic biology	Knockout of p53 family proteins	Demonstrated role of p53 family proteins in regulation and PSC differentiation	Wang et al. (2017)
HSPC	Basic biology	Ablation of CCR5	Efficient ablation with potential for hematopoietic cell-based therapy	Mandal et al. (2014)
iPSC	Basic biology	Gene silencing for genomic screening	Generation of a system for conducting genomic screens	Mandegar et al. (2016)
iPSC	Basic biology	Knockout of CHIP	Generation of CHIP knock out model useful in studying neurodegeneration	Schuster et al. (2019)
PSC	Basic biology	Genomic wide knockout	Demonstrated potential of CRISPR/Cas9 to perform genomic screens	Shalem et al. (2014)
iPSC	Alzheimer's disease	Cleavage of APP C-terminus	Proof of concept for development of APP silencing treatments	Sun et al. (2019)
iPSC	Cockayne syndrome	Gene correction of ERCC6	Demonstrated potential replacement therapy	Wang et al. (2019)
iPSC	RyR2 pathologies	Introduction of point mutations in hiPSCs	Facilitated mechanistic analysis and demonstrated potential therapeutic strategies	Wei et al. (2018)
hPSC	FOXG1 syndrome	Dose control of FOXG1	Demonstrated the potential of dose control as a therapeutic strategy	Zhu et al. (2019)
iPSC	Retinitis pigmentosa	Introduce missense mutation in PRPF8	Generation of retinitis pigmentosa disease model	Foltz et al. (2018)
hPSCs/organoids	Polycystic kidney disease	Knockout of podocalyxin	Generation of polycystic kidney disease model	Freedman et al. (2015)
iPSCs	Lung diseases caused by AEC2 dysfunction	Gene correction $SFTPB^{121ins2}$ mutation	Generation of AEC2 dysfunction model and regeneration potential	Jacob et al. (2017)
Organoid	Serrated colorectal cancer	Introduce modifications of serrated colorectal cancer	Generation of serrated colorectal cancer disease model	Lannagan et al. (2019)
MSC	Parkinson's disease	Induction of RAGE secreting cells	Generation of potential therapy	Lee et al. (2019)

(Table 1). These CRISPR/Cas9 PSC-derived isogenic models are then scaled up and differentiated into appropriate cell types or organoid cultures. One of the appeals of isogenic disease modeling is that these models lack the genetic variability of a patient-derived culture; hence, the gene of interest can be effectively studied without other confounding variables. Due to the high target specificity of the CRISPR/Cas9 system, the rest of the cell's genetic background remains intact (Ran et al. 2013). Furthermore, the PSCs' capacity to rapidly proliferate and differentiate remains unaffected by the CRISPR/Cas9 genomic modification (Ran et al. 2013; Zhang et al. 2017).

A number of groups have successfully utilized the CRISPR/Cas9 system to generate isogenic disease models. For instance, to model polycystic kidney disease, human PSCs were cultured to generate organoids. CRISPR/Cas9 was then incorporated to knockout polycystic kidney disease genes 1 and 2 inducing the formation of cysts (Freedman et al. 2015). To model aging and disease in vascular tissue, CRISPR/Cas9 was used to edit the expression of hypoxia-inducible factor 1α in human inducible PSCs (iPSCs), demonstrating its importance in disease and aging (Acun and Zorlutuna 2019). To model serrated colorectal cancer, organoid cultures were subjected to sequential CRISPR/Cas9-induced modifications associated with the disease and stem cell regulation. These modifications generated an adenocarcinoma model with a serrated colorectal cancer phenotype (Lannagan et al. 2019).

Perhaps, a more obvious strategy for modeling human disease in PSCs is to use PSCs derived from patient samples. Cultures generated from patient samples yield PSC-derived disease models which fully replicate the patient-specific genotype (Motta et al. 2017; Liu et al. 2019; Mohamed et al. 2019). These PSC-derived disease models are extremely useful in understanding the disease profile of a specific patient. However, their innate variability can make them unsuitable

for the broad study of a specific disease phenotype, and variability between PSC cultures from different patients is often observed (Mohamed et al. 2019). However, when utilized alongside CRISPR/Cas9-generated isogenic PSCs, they can provide insights into the causes of these variations. They can also be used to provide validation for CRISPR/Cas9-modified PSC-derived isogenic models. In a study exploring type 2 ryanodine receptor (RyR2) pathologies, CRISPR/Cas9 was used to induce point mutations in RyR2 human iPSCs, which were then differentiated into cardiomyocytes. When compared to patient-derived cells, the CRISPR/Cas9-modified isogenic PSC model had a comparable phenotype, thus validating the potential of the model (Wei et al. 2018).

CRISPR/Cas9-mediated gene correction in patient-derived PSC disease models

The concept of correcting disease-specific gene defects is an appealing one and CRISPR/Cas9 has the capacity to be utilized in an organism-wide scale. However, to ensure the potential of such an approach, the delivery of CRISPR/Cas9-mediated gene corrections in patient-derived PSC disease models provides much-needed insights into the potential of this approach on a large-scale in vitro. Through the design and development of gRNAs, CRISPR/Cas9 effectively breaks the DNA sequence near the target site and replaces the diseased DNA sequence with a corrected one (Zhang et al. 2014; Schmidt and Grimm 2015). The dysfunction of pulmonary alveolar epithelial type 2 cells (AEC2) is common in various types of lung disease. CRISPR/Cas9 was used to correct mutant genes in patient-specific PSC-derived AEC2 cells and was found to normalize AEC2 processing (Jacob et al. 2017). In umbilical cord blood-derived mesenchymal stem cells, CRISPR/Cas9 was used to edit soluble receptor for advanced glycation end products (sRAGE) secretions. The modified stem cells were then transplanted into a Parkinson's disease model, where they were shown to reduce neuronal loss (Lee et al. 2019). Similarly, in a patient-derived PSC model of retinitis pigmentosa, a common form of inherited blindness, CRISPR/Cas9, was used to correct missense mutations in genes associated with the condition. Following CRISPR/Cas9 correction, the corrected iPSCs were found to have a near normal phenotype (Foltz et al. 2018).

CRISPR/Cas9-modified PSCs can also provide invaluable insights into a disease-specific target gene as a potential therapeutic strategy. The potential for patient-derived PSC disease models and CRISPR/Cas9 to develop more personalized therapeutic approaches is particularly exciting as the response of the patient-derived disease model to the CRISPR/Cas9 modification will be specific to each patient's unique genotype. CRISPR/Cas9-mediated gene correction of the CSB/ERCC6 gene in Cockayne Syndrome, using patient-derived iPSCs, showed rescue of premature aging (Wang et al. 2019). In a study exploring the FOXG1 syndrome, CRISPR/Cas9 and small molecule-assisted shut-off (SMASh) technologies were incorporated in hPSCs to facilitate dose control of FOXG1. Regulation of FOXG1 was shown to influence cellular phenotype and demonstrated the potential of CRISPR/Cas9 as a tool for protein regulation in FOXG1 syndrome and other similar conditions (Zhu et al. 2019). CRISPR/Cas9-mediated cleavage of the C-terminus of APP in human iPSC-derived neurons revealed no effect of neuronal physiology, while attenuating beta-amyloid production (Sun et al. 2019), providing useful insights for the potential treatment of Alzheimer's disease.

Facilitation of PSC-mediated regenerative therapies and tissue repair

The rapid proliferative potential and capacity to differentiate into a multitude of specialized cell types make PSCs an ideal candidate for regenerative and/or replacement therapeutic strategies. When any kind of transplantation or regenerative approach is incorporated in vivo, the risk of the recipient rejecting the transplanted cells is significant (Morizane et al. 2013; Sohn et al. 2015; Zhang et al. 2017). Thus, using patient-derived PSCs may aid in the success of regenerative approaches. CRISPR/Cas9 has simplified and extended the scope of these approaches. In the case of regenerative therapies or tissue repair, CRISPR/Cas9 can be utilized to edit target DNA to stimulate transcription of necessary genes and/or stimulate PSC differentiation into the needed cell type (Chen et al. 2014; McKee and Chaudhry 2017). In one such approach, CRISPR/Cas9 was employed to target the promoter of ectodysplasin to induce the differentiation of mesenchymal stem cells into sweat gland-like cells (Sun et al. 2018). These CRISPR/Cas9-modified cells were found to differentiate into sweat gland-like cells. Upon transplantation into an animal model of deep burn injury, the sweat gland-like cells were capable of inducing perspiration (Sun et al. 2018), highlighting the potential for CRISPR/Cas9 in regenerative therapies.

Conclusions

To effectively understand normal and disease pathophysiology, PSC cultures provide an invaluable tool. Through their ability to rapidly proliferate, PSC cultures can be efficiently scaled up to generate the large-scale cultures needed to facilitate in-depth study of a target protein or DNA sequence. The integrity of PSC culture genotype and proliferative capacity is essential to their usefulness as an in vitro disease model. CRISPR/Cas9 has been shown to provide reliable (80% accuracy) and efficient genomic editing which preserves the

integrity of PSC culture and CRISPR/Cas9-modified PSC cultures can be rapidly scaled up to study the gene of interest. Since its release, CRISPR/Cas9 has been widely utilized in PSC-based research to advance the understanding of basic physiology, generate disease models, and provide insights into various therapeutic strategies. However, germline cell and embryo genomic editing using CRISPR/Cas9 pose a number of ethical hurdles, since they might be exploited for genetic enhancement of non-medically relevant human traits, such as athletic ability, intelligence, or height. Apart from ethical issues, there are still significant technical obstacles that prevent CRISPR/Cas9 from entering the clinic. There is need to evaluate its safety aspects for human trials and to achieve effective targeted delivery to the target cells in vivo. The risks of off-target or unwanted edits are still unknown. Another challenge for gene therapy is that there is still much to learn about the role of genes in most diseases. As CRISPR/Cas9 becomes more understood, advancements can be made with respect to improving target accuracy, meaning that these in vitro PSC studies have the capacity to be easily translated to in vivo preclinical and clinical studies and potentially improve the low clinical trial success rate that dominates today's research.

Acknowledgements This work was supported by the Zhejiang Provincial Science and Technology Projects (no. LGF19H060005 to Q. N. Y.).

Author contributions All authors have participated equally in drafting and revising this paper.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

Acun A, Zorlutuna P (2019) CRISPR/Cas9 edited induced pluripotent stem cell-based vascular tissues to model aging and disease-dependent impairment. Tissue Eng Part A 25:759–772. https://doi.org/10.1089/ten.tea.2018.0271

Anders C, Niewoehner O, Duerst A, Jinek M (2014) Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease. Nature 513:569–573. https://doi.org/10.1038/nature13579

Anderson EM, Haupt A, Schiel JA et al (2015) Systematic analysis of CRISPR-Cas9 mismatch tolerance reveals low levels of off-target activity. J Biotechnol 211:56–65. https://doi.org/10.1016/j.jbiotec.2015.06.427

Barrangou R (2015a) Diversity of CRISPR-Cas immune systems and molecular machines. Genome Biol 16:247

Barrangou R (2015b) The roles of CRISPR-Cas systems in adaptive immunity and beyond. Curr Opin Immunol 32:36–41

Barrangou R, Doudna JA (2016) Applications of CRISPR technologies in research and beyond. Nat Biotechnol 34:933–941. https://doi.org/10.1038/nbt.3659

Batista PJ, Molinie B, Wang J et al (2014) M^6A RNA modification controls cell fate transition in mammalian embryonic stem cells. Cell Stem Cell 15:707–719. https://doi.org/10.1016/j.stem.2014.09.019

Brazelton VA, Zarecor S, Wright DA et al (2015) A quick guide to CRISPR sgRNA design tools. GM Crops Food 6:266–276. https://doi.org/10.1080/21645698.2015.1137690

Brouns SJJ, Jore MM, Lundgren M et al (2018) Small CRISPR RNAs guide antiviral defense in prokaryotes. HHS Public Access. 321:960–964. https://doi.org/10.1126/science.1159689.Small

Cao J, Wu L, Zhang SM et al (2016) An easy and efficient inducible CRISPR/Cas9 platform with improved specificity for multiple gene targeting. Nucleic Acids Res. https://doi.org/10.1093/nar/gkw660

Carroll D (2011) Genome engineering with zinc-finger nucleases. Genetics 188:773–782. https://doi.org/10.1534/genetics.111.131433

Chen KG, Mallon BS, McKay RDG, Robey PG (2014) Human pluripotent stem cell culture: considerations for maintenance, expansion, and therapeutics. Cell Stem Cell 14:13–26

Chen Y, Cao J, Xiong M et al (2015) Engineering human stem cell lines with inducible gene knockout using CRISPR/Cas9. Cell Stem Cell 17:233–244. https://doi.org/10.1016/j.stem.2015.06.001

Christian M, Cermak T, Doyle EL et al (2010) Targeting DNA double-strand breaks with TAL effector nucleases. Genetics 186:757–761. https://doi.org/10.1534/genetics.110.120717

Cornu TI, Thibodeau-Beganny S, Guhl E et al (2008) DNA-binding specificity is a major determinant of the activity and toxicity of zinc-finger nucleases. Mol Ther 16:352–358. https://doi.org/10.1038/sj.mt.6300357

Cox DBT, Platt RJ, Zhang F (2015) Therapeutic genome editing: prospects and challenges. Nat Med 21:121–131

Daley GQ (2012) The promise and perils of stem cell therapeutics. Cell Stem Cell 10:740–749

Ding Q, Lee YK, Schaefer EAK et al (2013a) A TALEN genome-editing system for generating human stem cell-based disease models. Cell Stem Cell 12:238–251. https://doi.org/10.1016/j.stem.2012.11.011

Ding Q, Regan SN, Xia Y et al (2013b) Enhanced efficiency of human pluripotent stem cell genome editing through replacing TALENs with CRISPRs. Cell Stem Cell 12:393–394. https://doi.org/10.1016/j.stem.2013.03.006

Doudna JA, Charpentier E (2014) The new frontier of genome engineering with CRISPR-Cas9. Science 346(6213):1258096

Doyon Y, Vo TD, Mendel MC et al (2010) Enhancing zinc-finger-nuclease activity with improved obligate heterodimeric architectures. Nat Methods 8:74

Engle SJ, Puppala D (2013) Integrating human pluripotent stem cells into drug development. Cell Stem Cell 12:669–677

Foltz LP, Howden SE, Thomson JA, Clegg DO (2018) Functional assessment of patient-derived retinal pigment epithelial cells edited by CRISPR/Cas9. Int J Mol Sci. https://doi.org/10.3390/ijms19124127

Freedman BS, Brooks CR, Lam AQ et al (2015) Modelling kidney disease with CRISPR-mutant kidney organoids derived from human pluripotent epiblast spheroids. Nat Commun. https://doi.org/10.1038/ncomms9715

Garneau JE, Dupuis M-È, Villion M et al (2010) The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. Nature 468:67–71. https://doi.org/10.1038/nature09523

Gasiunas G, Barrangou R, Horvath P, Siksnys V (2012) Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. Proc Natl Acad Sci 109:E2579–E2586. https://doi.org/10.1073/pnas.1208507109

Gutierrez-Guerrero A, Sanchez-Hernandez S, Galvani G et al (2018) Comparison of zinc finger nucleases versus CRISPR-specific nucleases for genome editing of the Wiskott-Aldrich syndrome locus. Hum Gene Ther 29:366–380. https://doi.org/10.1089/hum.2017.047

Hendel A, Bak RO, Clark JT et al (2015) Chemically modified guide RNAs enhance CRISPR-Cas genome editing in human primary cells. Nat Biotechnol 33:985–989. https://doi.org/10.1038/nbt.3290

Hockemeyer D, Wang H, Kiani S et al (2011) Genetic engineering of human pluripotent cells using TALE nucleases. Nat Biotechnol 29:731–734. https://doi.org/10.1038/nbt.1927

Hryhorowicz M, Lipinski D, Zeyland J, Slomski R (2017) CRISPR/Cas9 immune system as a tool for genome engineering. Arch Immunol Ther Exp (Warsz) 65:233–240. https://doi.org/10.1007/s00005-016-0427-5

Huang N, Huang Z, Gao M et al (2018) Induction of apoptosis in imatinib sensitive and resistant chronic myeloid leukemia cells by efficient disruption of bcr-abl oncogene with zinc finger nucleases. J Exp Clin Cancer Res 37:62. https://doi.org/10.1186/s13046-018-0732-4

Jacob A, Morley M, Hawkins F et al (2017) Differentiation of human pluripotent stem cells into functional lung alveolar epithelial cells. Cell Stem Cell 21:472–488. https://doi.org/10.1016/j.stem.2017.08.014

Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337(6096):816–821

Jinek M, East A, Cheng A et al (2013) RNA-programmed genome editing in human cells. Elife. https://doi.org/10.7554/elife.00471

Joung JK, Sander JD (2013) TALENs: a widely applicable technology for targeted genome editing. Nat Rev Mol Cell Biol 14:49–55. https://doi.org/10.1038/nrm3486.talens

Kim Y-G, Chandrasegaran S (1994) Chimeric restriction endonuclease (*Flavobacterium okeanokoites*/*Escherichia cofi*/hybrid restriction endonuclease/protein engineering/recognition and cleavage domains). Biochemistry 91:883–887

Kim YG, Cha J, Chandrasegaran S (1996) Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain. Proc Natl Acad Sci USA 93:1156–1160

Lannagan TRM, Lee YK, Wang T et al (2019) Genetic editing of colonic organoids provides a molecularly distinct and orthotopic preclinical model of serrated carcinogenesis. Gut 68:684–692. https://doi.org/10.1136/gutjnl-2017-315920

Lee J, Bayarsaikhan D, Arivazhagan R et al (2019) CRISPR/Cas9 edited sRAGE-MSCs protect neuronal death in Parkinson's disease model. Int J Stem Cells 12:114–124. https://doi.org/10.15283/ijsc18110

Li L, Wv LP, Chandrasegaran S (1992) Functional domains in *Fok* I restriction endonuclease. Biochemistry 89:4275–4279

Liu Y, Deng W (2016) Reverse engineering human neurodegenerative disease using pluripotent stem cell technology. Brain Res 1638:30–41

Liu B, Saber A, Haisma HJ (2019) CRISPR/Cas9: a powerful tool for identification of new targets for cancer treatment. Drug Discov Today 24:955–970. https://doi.org/10.1016/j.drudis.2019.02.011

Maeder ML, Thibodeau-Beganny S, Osiak A et al (2008) Rapid "open-source" engineering of customized zinc-finger nucleases for highly efficient gene modification. Mol Cell 31:294–301. https://doi.org/10.1016/j.molcel.2008.06.016

Makarova KS, Grishin NV, Shabalina SA et al (2006) A putative RNA-interference-based immune system in prokaryotes: computational analysis of the predicted enzymatic machinery, functional analogies with eukaryotic RNAi, and hypothetical mechanisms of action. Biol Direct 1:7. https://doi.org/10.1186/1745-6150-1-7

Makarova KS, Haft DH, Barrangou R et al (2011) Evolution and classification of the CRISPR-Cas systems. Nat Rev Microbiol 9:467–477. https://doi.org/10.1038/nrmicro2577

Mali P, Yang L, Esvelt KM et al (2013) RNA-guided human genome engineering via Cas9. Science (80-) 339:823–826. https://doi.org/10.1126/science.1232033

Mandal PK, Ferreira LMR, Collins R et al (2014) Efficient ablation of genes in human hematopoietic stem and effector cells using CRISPR/Cas9. Cell Stem Cell 15:643–652. https://doi.org/10.1016/j.stem.2014.10.004

Mandegar MA, Huebsch N, Frolov EB et al (2016) CRISPR interference efficiently induces specific and reversible gene silencing in human iPSCs. Cell Stem Cell 18:541–553. https://doi.org/10.1016/j.stem.2016.01.022

McKee C, Chaudhry GR (2017) Advances and challenges in stem cell culture. Colloids Surfaces B Biointerfaces 159:62–77

Merkert S, Martin U (2016) Targeted genome engineering using designer nucleases: state of the art and practical guidance for application in human pluripotent stem cells. Stem Cell Res 16:377–386. https://doi.org/10.1016/J.SCR.2016.02.027

Miller JC, Holmes MC, Wang J et al (2007) An improved zinc-finger nuclease architecture for highly specific genome editing. Nat Biotechnol 25:778–785. https://doi.org/10.1038/nbt1319

Mohamed N-V, Larroquette F, Beitel LK et al (2019) One step into the future: new iPSC tools to advance research in parkinson's disease and neurological disorders. J Parkinsons Dis. https://doi.org/10.3233/jpd-181515

Mohr SE, Hu Y, Ewen-Campen B et al (2016) CRISPR guide RNA design for research applications. FEBS J 283:3232–3238

Mojica FJM, Montoliu L (2016) On the origin of CRISPR-Cas technology: from prokaryotes to mammals. Trends Microbiol 24:811–820

Mojica FJM, Díez-Villaseñor C, García-Martínez J, Almendros C (2009) Short motif sequences determine the targets of the prokaryotic CRISPR defence system. Microbiology 155:733–740. https://doi.org/10.1099/mic.0.023960-0

Morizane A, Doi D, Kikuchi T et al (2013) Direct comparison of autologous and allogeneic transplantation of IPSC-derived neural cells in the brain of a nonhuman primate. Stem Cell Rep 1:283–292. https://doi.org/10.1016/j.stemcr.2013.08.007

Motta BM, Pramstaller PP, Hicks AA, Rossini A (2017) The impact of CRISPR/Cas9 technology on cardiac research: from disease modelling to therapeutic approaches. Stem Cells Int 2017:1–13. https://doi.org/10.1155/2017/8960236

Nakano C, Kitabatake Y, Takeyari S et al (2019) Genetic correction of induced pluripotent stem cells mediated by transcription activator-like effector nucleases targeting ALPL recovers enzyme activity and calcification in vitro. Mol Genet Metab 127:158–165. https://doi.org/10.1016/j.ymgme.2019.05.014

Nii T, Kohara H, Marumoto T et al (2016) Single-cell-state culture of human pluripotent stem cells increases transfection efficiency. Biores Open Access 5:127–136. https://doi.org/10.1089/biores.2016.0009

Ormond KE, Mortlock DP, Scholes DT et al (2017) Human germline genome editing. Am J Hum Genet 101:167–176. https://doi.org/10.1016/j.ajhg.2017.06.012

Ramirez CL, Foley JE, Wright DA et al (2008) Unexpected failure rates for modular assembly of engineered zinc fingers. Nat Methods 5:374

Ran F, Hsu P, Wright J et al (2013) Genome engineering using crispr-cas9 system. Nature protocols. Nature Publishing Group, London, pp 2281–2308

Rath D, Amlinger L, Rath A, Lundgren M (2015) The CRISPR-Cas immune system: biology, mechanisms and applications. Biochimie 117:119–128. https://doi.org/10.1016/J.BIOCHI.2015.03.025

Reubinoff BE, Pera MF, Fong C-Y et al (2000) Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol 18:399–404. https://doi.org/10.1038/74447

Reyon D, Tsai SQ, Khayter C, Foden JA, Sander JDJJ (2013) FLASH assembly of TALENs enables high-throughput genome editing. Nat Biotechnol 30:460–465. https://doi.org/10.1038/nbt.2170.FLASH

Schmidt F, Grimm D (2015) CRISPR genome engineering and viral gene delivery: a case of mutual attraction. Biotechnol J 10:258–272

Schuster S, Saravanakumar S, Schöls L, Hauser S (2019) Generation of a homozygous CRISPR/Cas9-mediated knockout human iPSC line for the STUB1 locus. Stem Cell Res. https://doi.org/10.1016/j.scr.2018.101378

Shalem O, Sanjana NE, Hartenian E et al (2014) Genome-scale CRISPR-Cas9 knockout screening in human cells. Science (80-) 343:84–87. https://doi.org/10.1126/science.1247005

Smith J (2000) Requirements for double-strand cleavage by chimeric restriction enzymes with zinc finger DNA-recognition domains. Nucleic Acids Res 28:3361–3369. https://doi.org/10.1093/nar/28.17.3361

Sohn EH, Jiao C, Kaalberg E et al (2015) Allogenic iPSC-derived RPE cell transplants induce immune response in pigs: a pilot study. Sci Rep 5:11791

Sterneckert JL, Reinhardt P, Schöler HR (2014) Investigating human disease using stem cell models. Nat Rev Genet 15:625–639

Sun S, Xiao J, Huo J et al (2018) Targeting ectodysplasin promotor by CRISPR/dCas9-effector effectively induces the reprogramming of human bone marrow-derived mesenchymal stem cells into sweat gland-like cells. Stem Cell Res Ther 9:1–10. https://doi.org/10.1186/s13287-017-0758-0

Sun J, Carlson-Stevermer J, Das U et al (2019) CRISPR/Cas9 editing of APP C-terminus attenuates β-cleavage and promotes α-cleavage. Nat Commun. https://doi.org/10.1038/s41467-018-07971-8

Suzuki S, Sargent RG, Illek B et al (2016) TALENs facilitate single-step seamless SDF correction of F508del CFTR in airway epithelial submucosal gland cell-derived CF-iPSCs. Mol Ther Nucleic Acids 5:e273. https://doi.org/10.1038/mtna.2015.43

Tadić V, Josipović G, Zoldoš V, Vojta A (2019) CRISPR/Cas9-based epigenome editing: an overview of dCas9-based tools with special emphasis on off-target activity. Methods. https://doi.org/10.1016/j.ymeth.2019.05.003

Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676. https://doi.org/10.1016/j.cell.2006.07.024

Thomson JA, Itskovitz-Eldor J, Shapiro SS et al (1998) Embryonic stem cell lines derived from human blastocysts. Science 282:1145–1147. https://doi.org/10.1126/science.282.5391.1145

Trounson A, McDonald C (2015) Stem cell therapies in clinical trials: progress and challenges. Cell Stem Cell 17:11–22. https://doi.org/10.1016/j.stem.2015.06.007

Urnov FD, Rebar EJ, Holmes MC et al (2010) Genome editing with engineered zinc finger nucleases. Nat Rev Genet 11:636–646

Vandamme TF (2014) Use of rodents as models of human diseases. J Pharm Bioallied Sci 6:2–9. https://doi.org/10.4103/0975-7406.124301

Vercoe RB, Chang JT, Dy RL et al (2013) Cytotoxic chromosomal targeting by CRISPR/Cas systems can reshape bacterial genomes and expel or remodel pathogenicity islands. PLoS Genet. https://doi.org/10.1371/journal.pgen.1003454

Wang Q, Zou Y, Nowotschin S et al (2017) The p53 family coordinates Wnt and nodal inputs in mesendodermal differentiation of embryonic stem cells. Cell Stem Cell 20:70–86. https://doi.org/10.1016/j.stem.2016.10.002

Wang S, Min Z, Ji Q et al (2019) Rescue of premature aging defects in Cockayne syndrome stem cells by CRISPR/Cas9-mediated gene correction. Protein Cell. https://doi.org/10.1007/s13238-019-0623-2

Wei H, Zhang XH, Clift C 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. https://doi.org/10.1016/j.ceca.2018.04.009

Wong CH, Siah KW, Lo AW (2019) Estimation of clinical trial success rates and related parameters. Biostatistics 20:273–286. https://doi.org/10.1093/biostatistics/kxx069

Zhang F, Wen Y, Guo X (2014) CRISPR/Cas9 for genome editing: progress, implications and challenges. Hum Mol Genet. https://doi.org/10.1093/hmg/ddu125

Zhang Z, Zhang Y, Gao F et al (2017) CRISPR/Cas9 genome-editing system in human stem cells: current status and future prospects. Mol Ther Nucleic Acids 9:230–241

Zhu W, Zhang B, Li M et al (2019) Precisely controlling endogenous protein dosage in hPSCs and derivatives to model FOXG1 syndrome. Nat Commun. https://doi.org/10.1038/s41467-019-08841-7