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Genome engineering in human pluripotent stem cells

Udit Parekh[1,4], Marianna Yusupova[2,4] and Prashant Mali[3]

Human pluripotent stem cells (hPSCs) hold great promise for modeling and recapitulation of human biological processes. Coupled with the advent of genome engineering tools, specifically the CRISPR–Cas9 systems, hPSCs have opened a multitude of possibilities in modeling human biology and creating novel cellular therapies. Here, we review the fundamentals of both technologies, and the wide-ranging present and future applications of genome engineering in hPSCs.

Addresses
[1] Department of Electrical and Computer Engineering, University of California San Diego, USA
[2] Division of Biological Sciences, University of California San Diego, USA
[3] Department of Bioengineering, University of California San Diego, USA

Corresponding author: Mali, Prashant (pmali@ucsd.edu)
[4] These authors contributed equally.

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Introduction

The study of human development and disease has relied extensively on the use of animal models. Although animal models have proven invaluable, they do not fully capture or recapitulate human biology. It has been estimated that approximately 1% of human genes do not have homologs in the mouse genome [1] and genetic knockout studies in mice have also found divergence from human phenotypes [2–6], highlighting the need for alternate methods of modeling. The discovery of pluripotent stem cells revolutionized the research of human biology by providing a path to overcome this limitation.

Although stem cells helped overcome one challenge, the controlled manipulation of genetic and other cellular elements remained difficult. Over the last decade, the development of remarkably versatile tools for genome engineering has overcome many of these challenges, creating opportunities to study the genetic and epigenetic bases of normal and disease development, drug efficacy, creation of cellular gene-therapies, and more through performing site-specific manipulations within the genome [7].

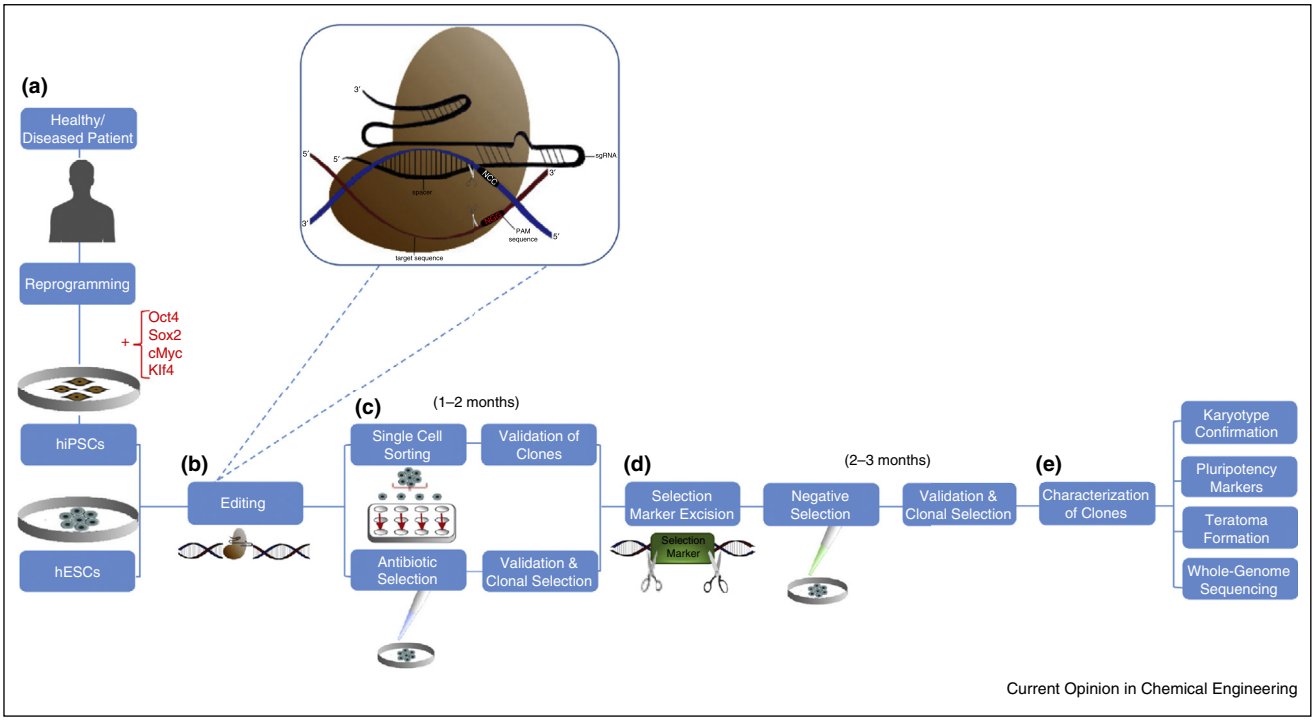
Pluripotent stem cells

Stem cells are capable of mitotically dividing to generate identical cell clones, or forming more specialized cell types through differentiation [1]. Different categories of stem cells are distinguished by their degree of potency, or capacity of differentiating into other cell types within the human body. Whereas unipotent or multipotent stem cells are self-renewing, their differentiation is limited to the cell types of the tissue from which they originate. By contrast, human pluripotent stem cells are self-renewing cells that have the capacity to develop into cell types of all three germ layers of the human body.

Previously, the term hPSCs primarily referred to human embryonic stem cells (hESCs), which could only be derived from the inner cell mass of the blastocyst during early embryonic development [1]. For this reason, obtaining such cells presented accessibility challenges and ethical issues involving their use. However, with the emergence of the paradigm-shifting iPSC technology in 2006 [2], adult somatic cells could be reprogrammed to have a stem cell fate through the expression of 4 critical transcription factors, Oct3/4, Sox2, c-Myc, and Klf4, in ESC culture conditions [2] (Figure 1a). The resulting iPSCs exhibit morphological and functional properties parallel to hESCs, having the potential to develop into tissues of all three germ layers as demonstrated by teratoma formation [2]. The quality and pluripotency of iPSCs is verified through karyotyping to ensure absence of chromosomal abnormalities, the detection of methylation patterns, and through the verification of pluripotency markers [2] (Figure 1e).

Additionally, iPSCs have allowed for production of functional cell types *in vitro* through employment of various stem cell differentiation methods, which can be used for experimental or potential therapeutic applications [3]. The virtual immortality of these cells in culture provides continuous resources for studying disease and development of differentiated cells types, while retaining the genomic information of the patient [4]. As human development requires highly precise levels of genetic control, leading to disease onset in the absence of regulation, the use of iPSCs enables studying of such processes that are otherwise difficult to recapitulate [5]. These properties of hPSCs have therefore enabled the study and modeling of early stages of human development and disease, both from patient-derived iPSCs as well as cell lines.

Figure 1



Overview of genome engineering workflow in hPSCs. Genome editing in hPSCs starts with the isolation of ESCs or reprogramming of adult somatic cells to hiPSCs. sgRNA, Cas9 and sequences for homologous recombination are delivered into the cell, separately or together to effect editing action. This is followed by isolation and validation of pure clones through antibiotic selection markers, single-cell sorting or both. If an antibiotic selection marker is used, it must then be excised so as not to interfere with normal expression of the gene. Finally, the engineered clonal cell line must be fully validated and characterized.

More recently, the self-organizing behavior of hPSCs has been exploited to form embryoid and organoid bodies, enabling the study of more complex developmental phenomena and tissue structures *in vitro* [8,9]. Similarly, it has also recently been demonstrated that three-dimensional microenvironments with defined chemical composition and biophysical characteristics can increase the efficiency and speed of induction of pluripotency [10•]. Thus, this provides a previously unexplored path to enhance not only differentiation but also the induction of pluripotency.

Genome editing/engineering

Genome editing experiments in mammalian cells about two decades ago [11,12] demonstrated that the introduction of a double-strand break in chromosomal DNA stimulated both non-homologous end joining (NHEJ) and homologous repair (HR) pathways, and increased the fraction of cells undergoing HR with an exogenously provided template by several orders of magnitude. This idea was made more powerful through development of site-specific nucleases: zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). Zinc finger nucleases combine the DNA recognition and binding properties of zinc finger proteins (ZFPs) with the cleavage properties of the nuclease domain of the FokI restriction enzyme [13]. TALENs also use the FokI nuclease, but instead of ZFPs, use proteins derived from highly conserved transcription activator like effector (TALE) repeat domains from *Xanthomonas* bacteria, which use these TALEs to affect transcription in host plants and promote infection [14]. Although ZFNs and TALENs are powerful tools for genome engineering, they must be reengineered for each editing site and are difficult to apply for engineering multiplex edits. The recent discovery and development of clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated systems, derived from bacterial adaptive immune systems [15], has overcome this drawback and significantly improved the ease of genome editing, leading to much more widespread application of genome engineering techniques.

The type II CRISPR system in bacteria functions through a RNA-guided mechanism, coupled with an associated effector nuclease, Cas9. In bacteria, the CRISPR–Cas9 system works in three stages. In the first stage, invading viral or plasmid DNA is cut and fragments incorporated into the CRISPR locus as 'spacer' sequences separated by repeat sequences. In the event of a repeat invasion, the

CRISPR locus is transcribed, and subsequently processed to form mature CRISPR RNA (crRNA) complexes. These complexes consist of the transcribed crRNAs of which the repeat regions are typically hybridized with trans-activating crRNAs (tracrRNAs) and associated with a Cas9 nuclease. The complex recognizes and locates the invading double-stranded DNA (dsDNA) sequences guided by the transcribed spacer region, and effects a DSB via the Cas9 effector nuclease [16].

Pioneering experiments demonstrated that the type II CRISPR–Cas9 system, derived from *Streptococcus pyogenes*, recognizes dsDNA sequences through simple base-pairing with the crRNA and the presence of an adjacent motif, called the protospacer adjacent motif (PAM), which aids in self versus non-self recognition [17]. It was found that the presence of both the crRNA and tracrRNA is required to effect cleavage by Cas9 and it was also demonstrated that a single chimeric guide RNA (sgRNA) combining the attributes of the tracrRNA and crRNA can be utilized to guide Cas9 cleavage. This makes the CRISPR–Cas9 system a versatile and powerful tool for programmable genome editing since it requires the production of a single RNA molecule to guide dsDNA cleavage via simple base-pairing, with a common effector nuclease.

Targeting of human pluripotent stem cells with CRISPR–Cas9

The most common types of genome manipulations performed using CRISPR–Cas9 include gene knockouts, or knock-ins through substitution of a target genetic sequence with a desired donor sequence (Figure 2a). Combined with hPSCs these may be used for functional genetic screening assays or *in vitro* recapitulation of a single gene or group of genes within the context of normal development, cellular differentiation, or progression of diseases with known genetic basis (Figure 2c).

Editing in absence of a homologous template

After the Cas9 nuclease creates a double-stranded cut at the target sequence of interest, DNA repair mechanisms are activated within the cell. In the absence of a homologous sequence that can provide a template for DNA repair, different pathways including microhomology-mediated end-joining (MMEJ), single-strand annealing, or non-homologous end-joining (NHEJ) can be activated after a double-stranded break occurs, although NHEJ is the most common pathway activated by the CRISPR–Cas9 system [18]. However, this pathway can lead to errors upon continuous activity by the Cas9 nuclease, generating point mutations or insertions-deletions (indel) mutations that result in reading frame shifts and disrupt protein expression when incorporated into exonic sequences of a gene [18,19]. Therefore, researchers may generate a gene knockout using CRISPR–Cas9 by targeting a specific and unique sequence within that gene, or disrupt an entire gene family by targeting a sequence common to the function of that group of genes [19]. Through multiplexed genome-targeting, in which multiple sgRNAs target different genes simultaneously, the role of multiple genes in a specific process can be evaluated through NHEJ disruption [20••]. Additionally, the NHEJ pathway can also be used to excise larger sections by using two sgRNAs targeting sequences that flank the section to be excised [21].

Editing through homology-directed repair (HDR)

A second common mechanism of DNA double-stranded break repair activated by CRISPR–Cas9, HDR, uses a homologous template to execute repair [22]. This is useful for precise genomic modifications through the introduction of exogenous templates, which can be incorporated during repair, such as for correction of a mutant gene copy with a normal copy, or generation of an isogenic cell line [23]. CRISPR–Cas9 editing through HDR has been utilized to create point mutations and small modifications using the introduction of single-stranded DNA templates [24,25••] or plasmid donor vectors, along with plasmids for sgRNA and Cas9 expression. Larger insertions can be achieved by utilizing donor templates with larger homology arms (up to 1 kb), albeit with low efficiency in hPSCs [26••], where efficiencies are typically of the order of 10^{-2} to 10^{-5} editing rates for those loci which yield correctly targeted clones.

These approaches are particularly useful for application with patient-derived iPSCs that contain known disease-associated mutation(s), which can be corrected (Figure 1b) and studied in parallel with the mutant counterparts or used for potential *in vivo* cell-replacement therapies [23] (Figure 2c). HDR modifications also enable engineering of hPSC lines with stable expression of antibiotic and drug selection markers, fluorescent proteins, and so on, through targeting of the AAVS1 safe harbor locus [27], or loci of relevant genes to create reporter cell lines for various lineages upon hPSC differentiation [28–30].

Design of CRISPR–Cas9 and sgRNA

The sgRNA employed in CRISPR–Cas9 genome editing is a fusion of the individual crRNA and tracrRNA found in bacteria. It consists of a constant portion which forms hairpin loops to assist the binding of Cas9, and a variable portion which guides sequence recognition and dsDNA binding [17,31]. The variable region is a 20 base-pair sequence complementary to the target region, with the additional requirement that the target region must be flanked at the 3′ end by a conserved PAM sequence — NGG in *S. pyogenes* derived systems [17] — 3 base pairs upstream of which the DSB is created (Table 1).

Table 1

Current considerations for editing hPSCs using CRISPR–Cas9 and future directions for improvement.

Consideration	Current status	Future directions
Pluripotent stem cell induction	Although reprogrammed to pluripotency, iPSCs can sometimes retain the epigenetic memory of their original state [108], which can affect their differentiation capacity [107]	Harnessing dCas9 based epigenetic regulation to modulate reprogrammed stem cell state
iPSC differentiation and maturation	Differentiation protocols exist for numerous cell types but show significant variation between cell lines, often low efficiencies and often do not achieve mature adult phenotypes [113]	Differentiation by forced expression of transcription factors [114] or by gene activation using dCas9 [101,102,104••,105] Generation of complex tissues and multiple lineages using organoid technology [8,115], tissue engineering approaches [116] and chimeric models [119,120]
CRISPR/Cas9 targeting specificity	Already low degree of off-target editing observed in hPSCs [37•,38•] — but minimization is of critical importance in modeling and therapeutic applications	Choice of optimal sgRNA using computational tools [31] Cooperative strategies using nickase Cas9 [45] or FokI-fused dCas9 [50,51] Rational engineering of Cas9 for improved specificity [52,53]
Extending target sequences	The commonly used S. pyogenes derived CRISPR/Cas9 system must have a 5′-NGG sequence downstream as the PAM which restricts the possible targetable sequences	Alternate CRISPR/Cas systems derived from other organisms [54,55] and engineered nucleases [58,59] with different PAM requirements.
Delivery	Nucleofection is currently the most efficient method to deliver CRISPR/Cas9 vectors in hPSCs [69] Delivery using viral vectors including lentiviral vectors and AAVs has also been successfully demonstrated. Lipofection has been demonstrated in a limited number of studies but is low efficiency [70]	Improved methods for electroporation, transfection and transduction in hPSCs, in particular non-integrating delivery approaches.
Editing efficiency	The efficiency of homologous recombination especially in hPSCs remains low — requiring long protocols for selection and purification of clonal cell lines	Inhibition of NHEJ pathway using small molecules [128] or gene silencing [129] Cell lines with inducible Cas9 for more efficient editing in modeling applications [25••,74] Fusing of enzymes with dCas9 [111,112]

Although in principle, the sgRNA functions through simple Watson-Crick base pairing, nuclease activity varies considerably depending on the specific sgRNA used, even within those targeting proximal sites in the same locus. Large screens have been employed to determine variation in nuclease activity due to sgRNA choice [32]. Factors including very low or high GC-content, targeting of the transcribed or non-transcribed strand, and sequence-dependent affinity for Cas9 were found to affect activity.

Although substantial off-target editing events have been reported with the CRISPR–Cas9 system in some mammalian cells [33–36], the rate of off-target editing has been found to be low in hPSCs [37•,38•,39•]. Although low, the minimization of off-target editing is crucial for therapeutic applications or generation of isogenic cell lines for modeling and functional investigation applications. Off target editing events arise since mismatches of 1–3 base pairs can be typically tolerated, especially in the PAM-distal region of the sgRNA sequence [33–36,40]. Additionally, some mismatches can be tolerated in the PAM sequence as well, with sites having 5′-NAG flanking the protospacer also being cleaved by the Cas9 complex [33,34]. These off-target events have been profiled using whole genome sequencing [37•,38•,39•], ChIP-seq [41–43], and new techniques like GUIDE-seq [44] and HTGTS [45] which detect DSBs with high sensitivity across the whole genome.

Optimization of the CRISPR–Cas9 system specificity

The specificity of CRISPR–Cas9 systems can be increased by multiple methods (Table 1). To increase the specificity of sgRNA targeting, a number of computational tools have been developed and are now publicly available, where optimal sgRNAs can be designed and off-target effects predictively assessed [31,46–48]. The concentration of Cas9 and sgRNA delivered can also be optimized to improve targeting specificity, although lower concentrations will also lead to lower on-target cleavage [33–35]. This fact has also been exploited to engineer a small-molecule inducible Cas9 which can be partially deactivated by removal of the molecule after allowing sufficient time for on-target modification [49]. Cooperative strategies that require paired sgRNAs to create a DSB, such as nickase variants of Cas9 [21,40] that cleave

only one strand, or fusions of nuclease-null Cas9 to a FokI nuclease [50,51], can substantially suppress off-target editing [45]. Although such strategies boost specificity, they come at the cost of reducing on-target editing. To overcome this, engineered variants of the nuclease have recently been demonstrated, which improve specificity by rational engineering of the Cas9 protein for minimal off-target binding [52,53].

One restriction with CRISPR–Cas9 targeting is the requirement of a specific PAM sequence adjacent to the protospacer in order for binding and cleavage by Cas9. This restricts the sites in the genome that can be targeted using this system, with 5′-NGG being the PAM for the type II system from *S. pyogenes*. To expand the range of sites that can be edited, CRISPR–Cas9-like systems from other species with different PAM sequences have been identified [54–57], and Cas9 variants engineered to have altered PAM requirements [58,59] (Table 1).

Delivery and efficiency

The delivery of the sgRNA/CRISPR–Cas9 and target donor vectors in hPSCs can be accomplished in multiple ways, including viral delivery and non-viral methods (Table 1). Viral delivery for CRISPR–Cas9 editing includes the use of self-inactivating lentiviruses (LVs) and adeno-associated viruses (AAVs) that each have unique features. Advantages of using LVs include their high packaging capacity of approximately 9 kb [60] that can deliver all the necessary components for HDR via CRISPR–Cas9, their ability to target both proliferating and non-dividing cells, and their lack of replication-competence through elimination of necessary viral genes [61,62]. However, as the consequences of continuous Cas9 expression from lentiviral integration is not fully understood, integrase-deficient lentiviruses (IDLVs) have also been developed, enabling transient expression of Cas9-CRISPR components that may help ensure long-term safety and stability of target cells [63]. In comparison to IDLVs, AAVs are highly used due to their relatively low immunogenicity which is particularly important for *in vivo* applications [64]. However, they have a smaller insert capacity of approximately 4.7 kb, and different serotypes can yield variable results [65–67].

Although viral delivery methods yield high levels of transfection in cell types that may otherwise be more difficult to target [68], non-viral delivery methods such as lipid-mediated transfection (lipofection) and electroporation have been optimized for increased efficiency. They have therefore become the most common and efficient *in vitro* delivery methods [69] in hPSCs [70], particularly electroporation, which enables the tuning of voltage and poring pulse parameters for optimal transfection. Increasing CRISPR–Cas9 targeting efficiency and improving delivery to hPSCs are key challenges to expanding the potential of these combined technologies.

Generating edited iPSC lines

Although the generation of cells with point mutations via indels from NHEJ or small insertions using HDR can be accomplished with reasonable efficiency in iPSCs, large insertions such as fluorescent reporter genes are low efficiency processes in hPSCs, as previously mentioned. Generation of clonal cell lines typically involves transfection or electroporation of the hPSCs with plasmids for the transient expression of the sgRNA, Cas9, and the donor vector.

The donor vector will often contain a selection marker gene to enable enrichment of cells where insertion has occurred. Once enriched, individual colonies can be isolated and cultured (Figure 1c). In the case of reporter insertion in endogenous loci, the selection marker must be removed to prevent interference with normal gene expression and expression of the reporter (Figure 1d), such as by flanking the selection marker with loxP or FRT sites and removing it after excision using Cre or Flp recombinase [71], or through the recently adapted piggyBac transposon [72,73] system which results in scar-free removal. If no selection marker is used, single cells can be sorted using fluorescence-activated cell sorting and cultured further for validation (Figure 1c). The development of synthetic biomaterials which enhance the self-renewal abilities of individual hESCs could be further extended to various hPSCs, making them more suitable for research requiring such genetic perturbations [74].

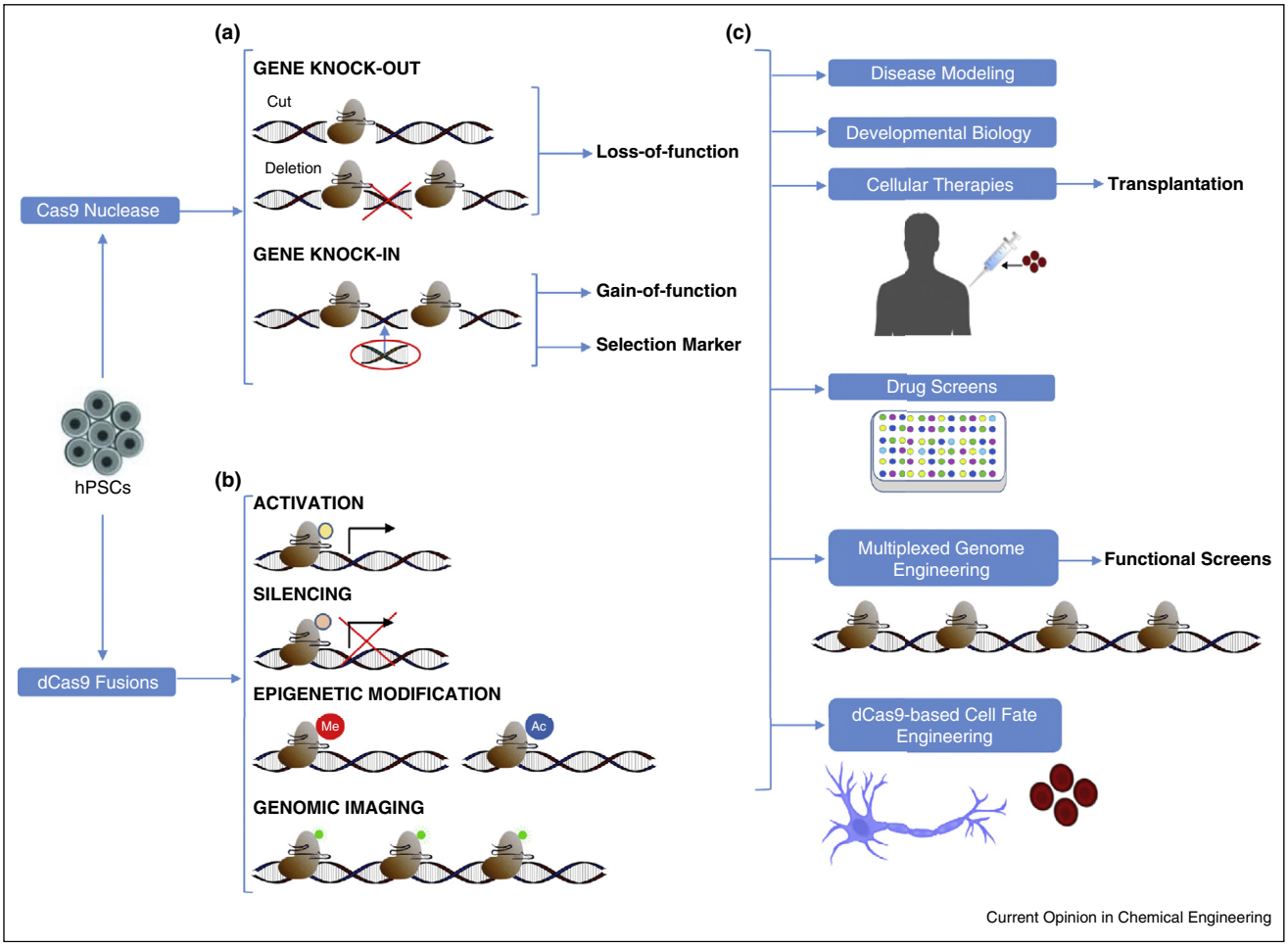
Recently, an inducible Cas9 system in hPSCs has been demonstrated, allowing higher efficiency HDR and hence selection-free clone generation [75], while exposure to low-dose irradiation has also been shown to significantly increase the rate of HDR [76]. Alternatively, NHEJ-mediated knock-in might also be employed, where the donor vector is composed of a reporter sandwiched between sgRNA target sites without any homology arms [77]. After the isolation of individual clones, each clone must be validated for accurate location of the indel or insertion, as well as for integration of the entire vector into the genome between the homology arms. This is most frequently accomplished via PCR, DNA sequencing methods and Southern Blots (Figure 1e).

Applications of iPSC genome engineering

Modeling development and disease

The CRISPR–Cas9 system can be combined with iPSCs to generate single or multiple gene knock-outs, correct mutations, or insert reporter transgenes. A number of studies have demonstrated mutations to have been corrected or introduced in iPSCs as a step toward therapy or for disease modeling. This includes the correction of mutations resulting in β-thalassemia [78], Duchenne muscular dystrophy [79], cystic fibrosis [80], deafness [81], and conferring resistance to HIV [82]. Multiple gene knockouts have been achieved using cell lines with

Figure 2



Applications of Genome Engineering in hPSCs. The application of CRISPR–Cas9 based genome engineering in hPSCs can be through editing to achieve gene knock-outs or knock-ins, for example, to correct disease causing mutations, generate reporter cell lines, or when used in a multiplexed form to generate large-scale genetic screens. Cas9 can also be used in its nuclease-null form (dCas9), fused with a diverse set of proteins for gene activation, gene repression, epigenetic modulation including methylation (Me) or acetylation (Ac), or fluorescent imaging of the genome. These diverse functions can be harnessed for modeling development and disease, engineering stem cell fate, and translational applications like drug screens and cellular therapies.

inducible Cas9 expression [25••] by fluorescent activated sorting of cells electroporated with sgRNAs, Cas9 and a fluorescent reporter [83], and by inducible Flp expression combined with CRISPR–Cas9 based insertion of FRT sites for deletion of sequences in exons [84]. Large deletions and rearrangements have also been enabled by inducing DSBs at two sites [85•], leading to the possibility of chromosomal engineering. In addition, such knock-outs can also be utilized to investigate epigenetic roles and targets, such as investigating DNA methylation by knocking out DNA methyltransferases [20••].

Genetic screens

The CRISPR–Cas9 system is also a powerful tool for large-scale, genome-wide screens. Although the RNA interference screens commonly used have proven effective, they have drawbacks, such as off-target effects, limitations in targeting only transcribed regions, and low signal-to-noise due to partial knockdowns.

In comparison, CRISPR–Cas9 screens [86] introduce a mutation in the genomic region of interest, causing frame-shifts in coding regions or mutagenesis of non-coding elements, resulting in permanent loss of function. These screens are typically performed by using pooled sgRNA libraries which can be delivered by lentiviral transfection after packaging together with Cas9, or separately or using Cas9 expressing cell lines. The cells are then screened by positive or negative selection (Figure 1c), and sgRNA presence or depletion quantitated by sequencing in a high-throughput, massively multiplexed manner with $>10^4$ sgRNAs and gene targets evaluated at a time

[32,87]. Such CRISPR screens have been employed in human cells to identify non-coding enhancer elements [88], identify cancer drug targets [87,89], unravel genetic pathways [32], identify essential genes [90] and fitness genes [91], study bacterial toxicity mechanisms [92], and immune regulatory networks [93].

Not only have CRISPR screens been employed to confer mutation induced loss of function, but by fusing transcription repressor or activator domains to nuclease-null Cas9 [94], loss of function screens can be enabled by CRISPR mediated repression or gain of function screens through CRISPR mediated activation [95,96].

Although few CRISPR screens have been performed on hPSCs so far, this potential is readily apparent. CRISPR screens could be combined with hPSCs to explore the genetic determinants of lineage choice, differentiation, and stem cell fate, providing the ability to evaluate the contributions of various genes or non-coding elements to specific processes and pathways (Figure 2c).

Expanding the CRISPR–Cas9 toolbox (dCas9 and beyond)

The versatility of the CRISPR–Cas9 system has been further harnessed through the creation of a null or 'nuclease-dead' Cas9 (dCas9), which has been rendered deficient of its enzymatic activity through mutation of the RuvC and HNH domains [94,97]. Since the dCas9 has retained programmability to target specific genomic loci in the presence of a sgRNA, it has been utilized as a recruitment molecule through fusion with other functional protein domains [40]. This therefore presents tremendous value by enabling controlled genetic regulation without introduction of permanent modifications to the sequence(s) of interest, along with other applications.

Transcriptional silencing

One such application of the dCas9 has been for transcriptional silencing in a system called CRISPR interference (CRISPRi) [94] (Figure 2b). This has been achieved through fusion of the dCas9 with a Kruppel-associated box (KRAB) repression domain to the N-terminus of the dCas9 (also referred to as KRAB-dCas9), which catalyzes heterochromatin formation [98]. This has been effective for knockdown of endogenous genes, as well as characterization of cis-regulatory elements that are important for transcription factor binding [94]. By utilizing a doxycycline-inducible TetO promoter rather than a constitutive promoter, the CRISPRi system has allowed for controlled and reversible gene silencing in iPSCs and iPSC-derived cells [99••]. Furthermore, as the CRISPRi induces changes to chromatin structure with high targeting specificity, it has been applied to epigenomic-level editing, including investigating the action of distal regulatory elements [98]. The combination of CRISPRi and iPSCs can further enable a new mode of genetic-screening, reversible disease modeling, and epigenomic-level regulation for deeper probing into developmental and differentiation pathways [98,99••] (Figure 2c).

Transcriptional activation

Similarly, the development of a dCas9-activator is of particularly great relevance, as it can enable manipulation of gene expression in highly-regulated transcriptional networks that are critical for early development, stem cell differentiation, and epigenetic control (Figure 2b). Engineering of a dCas9-activator has been achieved through fusion of the dCas9 C-terminus with the VP64 activator domain in human cells [40]. The VP64 activator domain functions as a scaffold that recruits various components of the transcription pre-initiation complex, therefore increasing transcriptional activation [97]. Additionally, the strength of transcriptional activation has also been optimized by fusing different combinations or multiple activator domains to the dCas9 [100]. In mouse embryonic fibroblasts (MEFs), fusion of one VP64 domain to both the N-termini and C-termini enabled enhanced transcriptional activation and differentiation [101], and multiplexed activation of various genes enabled chromatin remodeling and induction of neuronal fate [102]. Such results show the remarkable ability of dCas9-activators to induce transcription despite the presence of repressive chromatin marks. The sensitivity of the dCas9 activator system was also demonstrated in HEK293T cells where transcription of multiple endogenous genes was induced through simultaneous targeting of multiple proximal promoter sites [103].

These genetic regulation tools have also been applied for differentiation of hPSCs, such as into endodermal [104••] and neuronal lineage [105] (Table 1), and has also successfully induced gene expression in the presence of repressive epigenetic markers in hESCs, resulting in roughly a 290-fold increase in expression of certain genes [106].

Epigenetic regulation

Although activation domains fused to the dCas9 can indirectly affect the epigenetic state by targeting the promoter region, they do not catalytically modify the chromatin structure itself and may therefore be less effective in conferring larger scale or durable epigenetic changes. Similarly, specific dCas9-transcription factors utilized have been limited to inducing gene expression by targeting promoter regions [97]. Developing a deeper understanding of epigenetic control therefore requires examination and characterization of individual or combinations of epigenetic marks. To address this experimentally, researchers have created a dCas9 fused to the catalytic histone acetyltransferase (HAT) core domain of the human E1A-associated p300 acetyltransferase

(p300-core) to target proximal, core, and distal enhancers of the MYOD locus, thereby creating a means of epigenomic regulation at these various enhancer elements [97]. Ultimately, such an ability to modify epigenomic states in addition to upregulation and downregulation of specific genes is imperative in elucidating our understanding of gene regulatory networks [100]. Additionally, as iPSCs have been observed to retain an epigenetic memory which affects their differentiation capacity [107], such as through residual methylation marks corresponding to their tissue of origin [108], the manipulation of chromatin state via dCas9 and appropriate fusion domains can potentially reset the resulting influence on iPSC differentiation potential. Additionally, these Cas9-fusion proteins could be further utilized to provide a mechanism of developing screens for epigenetic regulation factors (Figure 2b).

Additional dCas9 applications

More recently, dCas9 has been extended beyond gene regulation and epigenomic modifications to chromosomal imaging (Figure 2b). Through the use of an EGFP-tagged dCas9 and structural optimization of the sgRNA molecule, researchers have enabled imaging of functional genes as well as telomere dynamics during various processes such as elongation, disruption, and mitosis [109]. This concept was further expanded upon in the development of the CRISPRainbow system, which has utilized the dCas9 to enable 3-D fluorescent labeling of genomic structure in living cells through the binding of engineered sgRNA scaffolds to fluorescent proteins of various colors [110••]. Although this novel system using the dCas9 has not been tested in hPSCs, such applications would be critical for gaining a larger chromosomal-scale understanding of various biological processes, or the role of chromosomal structure and stability in various diseases.

Finally, dCas9 may also offer a route to more efficient genome editing. Two recent studies have shown that dCas9 fused with a cytidine deaminase led to efficient point substitutions of cytidine with uracil, which has the base-pairing properties of thymine in the neighborhood of the sgRNA [111,112]. Similar fusions to enable more efficient HR can be projected.

Future directions

The combination of hPSCs and genome engineering using CRISPR–Cas9 is poised to deliver major advances in our understanding of development and disease, and advance technology for cell therapies. However, many challenges such as increasing the efficiency of genome editing in hPSCs remain, especially for HR, which is often dominated by the NHEJ pathway. The isolation of correctly targeted cells and removal of any drug-selection markers for generation of pure clonal cell lines is often a months-long process, also posing limitations.

Differentiation and maturation of hPSCs to adult phenotypes is still underdeveloped and advances will be required to model and treat adult disease [113]. Promising *in vitro* methods include differentiation through forced expression of transcription factors [114]. Although *in vitro* differentiation techniques are invaluable, stem cell differentiation *in vivo* is governed by the stem cell niche which includes cell–cell and cell–matrix interactions. Recently organoid technologies [8,115] which take advantage of stem cell self-organization, and tissue engineering approaches [116], which can combine stem cell self-organization with extra-cellular matrices and cues, have made strides toward recapitulating tissue function *ex vivo*. These methods currently rely primarily on animal-derived matrices which have variable compositions and are poorly defined, making them unsuitable for finely controlled investigations. The development of synthetic matrix materials [117•,118] which can be modified in a controlled manner will be crucial in unraveling the mechanisms regulating stem cell fate, developing better methods of differentiation and recapitulating organ function. Another promising approach is through chimeric models where hPSCs or differentiated cells are introduced into immune deficient animals for maturation [119,120].

Ameliorating the challenges associated with combining the CRISPR–Cas9 system and hPSCs will lead to many powerful applications. The ease with which sgRNAs can be designed and constructed make the CRISPR–Cas9 system ideally suited for large scale genome-wide screens, although they are yet to be implemented in hPSCs. Such screens will likely prove an invaluable tool, where inducible Cas9 systems triggered by drugs and small molecules [49] or optical stimulation [121,122] could give us exquisite spatiotemporal control. The use of these screens as well as knock-out, transcription regulation, genome imaging, and epigenetic perturbation studies will likely also lead to an improved understanding of the complex mechanisms underlying stem cell fate, the effect of perturbations on disease, and the discovery of new therapeutics. This could be further coupled with engineering approaches to modulate extra-cellular factors like the mechanical and chemical properties of the matrix [123,124].

Finally, as disease-causing mutations have been corrected in multiple cases in cell cultures [78–80] in the laboratory, they could similarly be edited for later infusion or transplantation, even to form complex structures by coupling with engineering approaches to add complexity and vascularization [125–127], and utilizing engineered materials which avoid animal derived products which can lead to adverse immune response. This illustrates the vast potential for the combination of hPSCs and genome engineering to have a catalytic effect on regenerative medicine, and transform our understanding of human biology.

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