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REVIEW

Manipulating and studying gene function in human pluripotent stem cell models

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(Received 1 June 2023, revised 4 July 2023, accepted 5 July 2023)

doi:10.1002/1873-3468.14709

Edited by Ivan Sadowski

Human pluripotent stem cells (hPSCs) are uniquely suited to study human development and disease and promise to revolutionize regenerative medicine. These applications rely on robust methods to manipulate gene function in hPSC models. This comprehensive review aims to both empower scientists approaching the field and update experienced stem cell biologists. We begin by highlighting challenges with manipulating gene expression in hPSCs and their differentiated derivatives, and relevant solutions (transfection, transduction, transposition, and genomic safe harbor editing). We then outline how to perform robust constitutive or inducible loss-, gain-, and change-of-function experiments in hPSCs models, both using historical methods (RNA interference, transgenesis, and homologous recombination) and modern programmable nucleases (particularly CRISPR/Cas9 and its derivatives, i.e., CRISPR interference, activation, base editing, and prime editing). We further describe extension of these approaches for arrayed or pooled functional studies, including emerging single-cell genomic methods, and the related design and analytical bioinformatic tools. Finally, we suggest some directions for future advancements in all of these areas. Mastering the combination of these transformative technologies will empower unprecedented advances in human biology and medicine.

Keywords: arrayed and pooled screens; base and prime editing; CRISPR interference and activation; CRISPR/Cas9; genomic safe harbors; homologous recombination; human pluripotent stem cells; RNA interference; single-cell screens; transgenesis

Abbreviations
AAVS1, adeno-associated virus integration site 1 (locus); amiRNA, artificial miRNA; ASO, antisense oligonucleotide; BE, base editing; Cas, CRISPR-associated protein; *CLYBL*, citrate lyase beta-like (locus); CRISPR, clustered regularly interspaced short palindromic repeats; CRISPRa, CRISPR activation; CRISPRi, CRISPR interference; CRISPRn, CRISPR nuclease; DHFR, dihydrofolate reductase; Dox, doxycycline; DSB, double-strand break; ESC, embryonic stem cell; GoF, gain of function; GOI, gene of interest; GSH, genomic safe harbor; HDR, homology-directed repair; hiPSC, human-induced pluripotent stem cell; hPSC-CM, human pluripotent stem cell-derived cardiomyocyte; HR, homologous recombination; *hROSA26*, human orthologue of the mouse Rosa26 (locus); KD, knockdown; KI, knockin; KO, knockout; LoF, Loss of function; MCP, MS2 capsid protein; miRNA, microRNA; modRNA, (chemically) modified mRNA; nCas9, Cas9 nickase; NHEJ, non-homologous end joining; OHT, hydroxytamoxifen; OPTiKD, optimized inducible knockdown; OPTiKO, optimized inducible knockout; PB, piggyBac (transposon); PE, prime editing; pegRNA, prime editing guide RNA; PuroR, Puromycin resistance marker; RNAi, RNA interference; rtTA, reverse tetracycline-controlled transactivator; sc-RNA-seq, single-cell RNA sequencing; sgRNA, single-guide RNA; shRNA, short hairpin RNA; siRNA, short interfering RNA; ssODN, single-stranded oligodeoxynucleotide; TALEN, transcription activator-like effector nuclease; Tet, tetracycline; TetR, tetracycline repressor protein; TF, transcription factor; TMP, trimethoprim; TRE, tetracycline-responsive element (promoter); ZFN, zinc finger nuclease.

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Human pluripotent stem cells (hPSCs) possess the extraordinary ability to both self-renew and differentiate into any somatic cell type. hPSCs can be obtained either from the inner cell mass of the developing blastocyst (embryonic, hESCs [1]) or from the reprogramming of somatic cells using Yamanaka factors (induced pluripotent, hiPSCs [2]). hPSCs offer unprecedented opportunities to study human development, investigate human diseases, and develop novel cell therapies to cure pathological conditions (reviewed in [3–5]). The full potential of all these applications, however, can only be realized through efficient manipulation of gene function in hPSCs and/or hPSC-derived cells. Perturbing gene expression is a fundamental tool when studying developmental and disease mechanisms. The application of hPSCs into the clinic does not in principle require altering their gene expression, but such an approach can be invaluable to overcome immune rejection or improve the safety of cell therapy [6–8]. The concept of combining cell and gene therapy has also recently taken center stage [9,10]. Last but not least, hPSC "forward programming"—forced differentiation into somatic cells through overexpression of cell fate regulators, such as transcription factors (TFs)—offers the potential to scalably and reproducibly generate mature cells for all aforementioned applications [11,12].

In this review, we aim to comprehensively cover the main approaches that can be utilized to manipulate gene function in hPSC models with varying levels of throughput, highlighting their pros and cons. We begin by highlighting the specific challenges associated with working with hPSCs. We then summarize the main approaches to deliver nucleic acids in hPSCs, followed by an overview of the most common methods that have been used to manipulate gene function in hPSC and hPSC-derived cells. A central focus is then posed on functional genomic approaches to study many perturbations in the same experiment, most recently through the use of single-cell genomics. We conclude with an outlook on existing limitations and some ideas for potential solutions.

Challenges with manipulating gene function in hPSC models

Human pluripotent stem cells are challenging to work with, particularly for those that are used to more sturdy immortalized cell lines. Compared to the early days of hPSC culture and differentiation, which relied on homemade growth matrices and media, the current availability of commercial options greatly facilitates the task. Reproducible cultures of high-quality hPSCs, however, do not overcome some key challenges intrinsic to this cell type. Of note, these challenges are distinct for "conventional" hPSCs, which are stabilized in the so-called "primed" pluripotent state, which recapitulates the post-implantation epiblast, and "naïve" hPSCs, which instead are coaxed to resemble the inner cell mass of the blastocyst [13]. We focus on conventional hPSCs, as most laboratories use culture conditions that are conducive to such a state.

hPSCs are delicate

Given the crucial importance of the pluripotent state to human development, it is perhaps unsurprising that its artificial stabilization *in vitro* is a delicate balance: even the best hPSC culture is characterized by a substantial degree of cell death. hPSCs are highly sensitive to metabolic changes, fluctuations in growth factor levels, shear stress, and both loss of cell contact and over-confluency. hPSCs are also extremely sensitive to genotoxic damage: mutations in the p53 pathway that desensitize cells from such an insult are commonly acquired in hPSC lines, particularly following long-term and/or suboptimal culture [14]. Recurrent chromosomal abnormalities leading to a proliferative advantage, such as isochromosome 20q, are similarly selected for [15]. Epigenetic modifications, such as gene hypermethylation and silencing, can also be acquired [16]. In female hPSCs, erosion of X chromosome inactivation is common [17]. The pro-apoptotic nature of hPSCs can be mitigated by the inhibition of Rho-associated protein kinase (ROCK) [18], but this strategy should not be abused as it affects hPSC metabolism and differentiation [19].

hPSCs are poorly clonogenic

The pluripotent epiblast is a columnar epithelium. hPSCs recapitulate this structure by growing in tightly compacted colonies that are normally passaged as clumps of multiple cells. Seeding of individual hPSCs to generate clonal populations is highly inefficient, as cells generally undergo anoikis (cell death due to lack of neighboring contacts). Besides optimization of cell dissociation, sorting, and replating methods, survival can be improved by adding growth supplements that contain, for instance, antioxidants, ROCK inhibitors, caspase inhibitors, and stress response inhibitors [20]. Clonal growth exacerbates the tendency to select hPSCs with genetic and epigenetic abnormalities: assessment of (epi)genome integrity is pivotal, as is the analysis of more than one clone to confirm the reproducibility of phenotypes.

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hPSCs silence many foreign sequences

Pluripotent cells must protect the soma from damaging mutations. This is partly achieved through the hypersensitivity to genotoxic DNA damage and in part by very efficient mechanisms that recognize and silence both exogenous and endogenous genetic parasites, such as viruses and transposons [21]. As discussed in further detail below, this strongly limits the efficacy of viral transduction. hPSCs even silence viral promoters in isolation, such as the otherwise strong CMV promoter [22]. Transgenic cassettes can be further silenced during hPSC differentiation. As cell fate specification restricts the developmental potential, many genomic regions become heterochromatic: transgenes contained within become transcriptionally inactive [23]. Moreover, promoters designed to work in proliferating cells, such as the commonly used tetracycline-responsive element (TRE), can lose their potency in differentiated post-mitotic cells [24,25].

Pluripotency complicates the manipulation of gene function

The study of gene function in hPSCs is challenged by the need to maintain such cells in a self-renewing undifferentiated state. Increased or decreased expression of genes implicated in pluripotency, proliferation, and survival is often incompatible with maintaining healthy hPSCs [26]. Moreover, most methods for efficient genetic manipulation of hPSCs require lengthy clonal selection procedures: it can be difficult to distinguish between the immediate effects of any given genetic manipulation and its potential indirect downstream effects. Finally, studying gene function at a specific stage of hPSC differentiation is complicated if such a gene is involved in multiple steps during lineage specification. Overall, experiments relying on stable loss- or gain-of-function in hPSCs can be complicated to execute and/or interpret. In such cases, it is pivotal to rely on more complex methods to conditionally manipulate gene expression, such as those based on transcriptional repressors or activators responsive to tetracycline (Tet) or its derivative doxycycline (Dox; i.e., Tet-On and Tet-Off; [27]), or recombinase-dependent approaches (i.e., Cre-lox; [28]).

Methods to deliver nucleic acids in hPSC models

Human pluripotent stem cells are not easily manipulated using traditional methods that work well on less sensitive cell lines [29–31]. Transient expression of nucleic acids in hPSCs is complicated by their rapid dilution through proliferation. Therefore, stable modification of hPSCs with exogenous DNA is usually the preferred approach. There are multiple ways by which this can be accomplished, each coming with its own set of pros and cons. We cover those most commonly applied to hPSCs (Fig. 1).

Plasmid integration

Plasmids are by far the easiest tool available to most laboratories. There are, however, some important aspects to consider when applying them to hPSCs. hPSCs are extremely sensitive to endotoxin: this should be minimized through preparatory purifications. Both chemical- and liposome-based transfection can be effective (particularly stem cell-specialized formulations), but liposomes are more sensitive to protein content in the media [32,33]. Transfection works best in very small clumps of exponentially growing hPSCs. This requires optimization of cell density versus the amounts of delivery agent and plasmid, so as to balance efficient delivery with minimal toxicity. While transfection of hPSCs remains relatively inefficient compared to other cell types, it can reach at least 30% efficiency in most hPSC lines [30]. Electroporation can introduce plasmids with similar or even higher efficiency [29], particularly through so-called nucleofection using stem cell-optimized solutions and pulse programs. However, it is associated with stronger toxic effects, partially due to the need to generate a single-cell suspension in order to maximize delivery. In our experience, plasmids larger than 10–15 kilobase pairs are challenging to deliver with either method.

Plasmids can be randomly integrated into the hPSC genome (Fig. 1A; [34–36]). This process is facilitated by prior linearization of the construct to trigger NHEJ [37]. Isolation of stably transfected or electroporated hPSCs can be simplified through markers, including fluorescent proteins and/or genes encoding resistance against antibiotics such as Puromycin, Blasticidin, Hygromycin, or Neomycin (antibiotics listed in order of our preference: Neomycin is particularly challenging to work with given the narrow efficacious concentration range [38]). Importantly, selective marker expression should be ideally operationally linked to the modification of interest (i.e., as part of a polycistronic construct): otherwise, given the tendency of hPSCs to silence foreign DNA, it is possible to select cells only expressing the selective marker but not the rest of the transgenic cassette. Along the same lines, maintained selective pressure may be needed to prevent silencing of the randomly integrated plasmid, which is further

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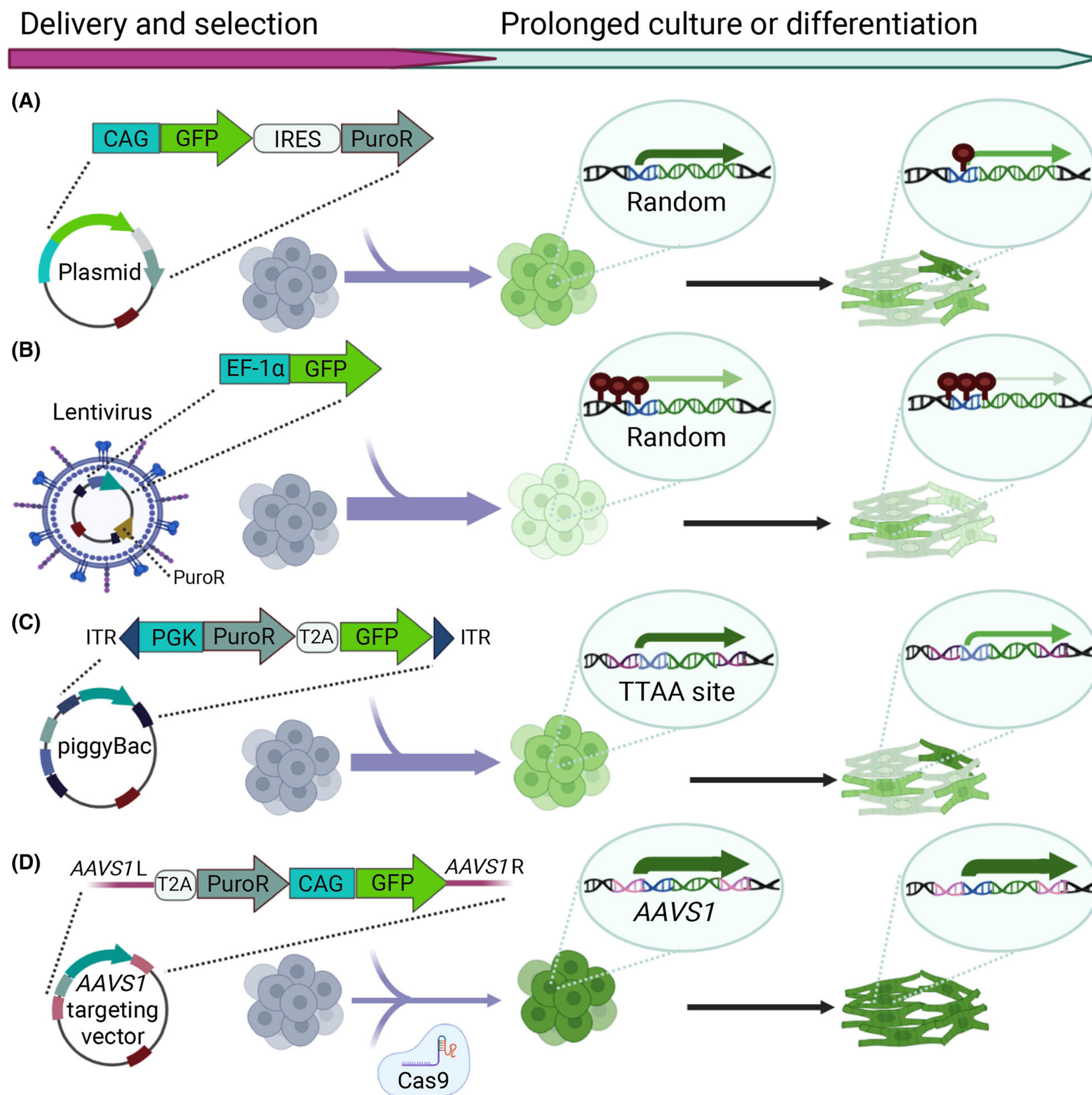

Fig. 1. Stable delivery of DNA in hPSC models. Representative methods are contrasted by delivery efficiency (thickness of blue arrow) and activity in hPSCs and hPSC-derived cells (thickness and intensity of green arrow). A selective marker (i.e., puromycin N-acetyl-transferase, PuroR) allows selection of hPSCs that stably integrate the cargo DNA. (A) Plasmid delivery (i.e., pTP6 transfection [35]) has modest efficiency and the random integration is subject to positional effects before and/or after differentiation. (B) Lentiviral transduction (i.e., FUGW [46]) is very efficient but subject to silencing of random integrations. (C) Transposition (i.e., piggyBac transfection [179]) is more efficient than random plasmid integration. (D) Genome editing (i.e., CRISPR/Cas9 editing of *AAVS1* genomic safe harbor [61]) is inefficient but expression is reproducible and stable after differentiation. CAG, EGF-1α, PGK: constitutive Pol II promoters; GFP: green fluorescent protein; IRES: internal ribosome entry site; ITR: inverted tandem repeat; T2A: self-splicing viral peptide; *AAVS1* L/R: left and right homology arm.

subjected to the possibility of silencing and/or expression variegation following hPSC differentiation. If working on clonal lines, it is likely to observe differences in the potency of the modification due to positional effects of the insertions [39–42]. It is accordingly hardly possible to exactly reproduce the same modification in more than one hPSC line. Plasmids can also be transfected after hPSC differentiation, but the efficiency of

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such an approach is highly dependent on the specific lineage [43].

Viral integration

Human pluripotent stem cell transduction can be quite efficient, but, as already mentioned, hPSCs rapidly and efficiently silence most common viral vectors [23,44–46]. This property is actually key to the generation of hiPSCs through retroviral or lentiviral overexpression of Yamanaka factors in somatic cells, which relies on the silencing of the transgenes after the completion of reprogramming [2,47]. Nevertheless, many laboratories have performed lentiviral transduction of hPSCs, given the wide availability and relative ease of use of these reagents (Fig. 1B). Selection of transduced hPSCs can be achieved by similar means to those described above for plasmid delivery, but it is subject to an even stronger tendency toward silencing over passaging and/or differentiation. The careful choice of promoters and other elements of the transgenic cassette becomes even more paramount. For instance, promoters such as EF-1alpha or PGK are less prone to silencing compared to CMV [46,48]. Derivation of transfected clones that stably and strongly express the transgenic cassette is possible, but it can be laborious and poorly reproducible. Therefore, lentiviral transduction of hPSCs is more suitable for experiments that rely on short-term readouts not substantially affected by silencing. Lentivirus can alternatively be utilized after hPSC differentiation to bypass silencing in the pluripotent stage. In some lineages, however, alternative viral vectors may prove more efficient, including non-integrating viruses discussed below [49,50].

Transposition

The piggyBac (PB) transposon is particularly suited to the delivery and/or removal of nucleic acids in hPSCs [51], being more efficient than less common alternatives such as Sleeping Beauty [52]. PB integrates a cargo surrounded by inverted terminal repeats at TTAA sites and leaves no footprint after its excision. Integration and excision of PB lacking the transposase gene can be controlled by transiently expressing transposase. This property has been leveraged to reprogram somatic cells into transgene-free hiPSCs through transient expression of reprogramming factors *via* PB [53]. PB can carry very large cargoes up to hundreds of kilobase pairs, are less sensitive to silencing compared to lentiviral vectors, and are more efficiently integrated into the genome compared to standard plasmids. Thus, they are particularly useful for introducing multiple perturbations at once in a population of hPSCs [54]. PB is usually delivered within a plasmid, and its successful transposition in hPSC can be selected for using markers expressed by the cargo (Fig. 1C). PB preferentially integrates into transcribed regions, but remains susceptible to silencing during differentiation due to chromatin remodeling mechanisms.

Gene editing

Programmable nucleases

Gene editing based exclusively on homologous recombination (HR) is quite ineffective in hPSCs, and is further complicated by their limited clonogenicity [55]. This situation was dramatically changed following the development of efficient gene editing tools such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs; reviewed in [56,57]). These methods are based on customizable DNA binding domains engineered to recognize specific sequences and fused to nucleases that induce site-specific double-strand DNA breaks (DSBs). Such events can be repaired by the error-prone non-homologous end joining (NHEJ) pathway, resulting in random small insertions or deletions (indels) that can generate null alleles. Alternatively, homology directed repair (HDR) mechanisms can drive HR of a donor DNA fragment carrying specific mutations, resulting in precise gene editing (reviewed in [58,59]).

More recently, this toolbox was further expanded by modified prokaryotic type-II clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated protein 9 (Cas9) systems, whereby a Cas9 endonuclease is selectively targeted to a genomic locus by a single-guide RNA (sgRNA) through Watson–Crick base pairing [60–62]. Given its simplicity, efficiency, and flexibility, CRISPR/Cas9-mediated gene editing has quickly become the gold standard for genetic manipulation of multiple cell types, including hPSCs (reviewed in [63]). Moreover, variations of traditional CRISPR/Cas9 systems that are based on fusion proteins with catalytically inactive Cas9 allow a wide range of targeted applications, including transcriptional interference or activation [64–66].

Programmable nucleases have been widely used to engineer hPSCs, as described in more detail in the following sections. However, compared to other established cell types, it is paramount to keep in mind the hypersensitivity of hPSCs to genotoxic damage: induction of DSBs by any nuclease frequently leads to p53 activation and apoptosis [14,67,68]. Cells that survive

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the genome editing process are often enriched for mutations in this pathway and should be carefully evaluated for genetic and epigenetic abnormalities that can lead to a proliferative and/or survival advantage, particularly following clonal isolation. Another important factor to consider is that hPSCs have low intrinsic HDR activity, while NHEJ is generally more active [69]. The efficiency of HR can be maximized by using exponentially growing cells cultured at low confluency prior to editing (HDR is most active in S and G2), or by the addition of small molecule inhibitors of NHEJ [70].

Genomic safe harbors

Another major advance in hPSC gene editing was the identification of human genomic safe harbors (GSHs): regions in the genome that are active in virtually any human cell type, are resistant to gene silencing and can be genetically modified without negatively affecting cellular functionality [71]. GSHs are attractive options for robust genetic engineering of hPSCs, as they can mitigate silencing both in the undifferentiated state and after differentiation. The most popular GSH for hPSC engineering is the so-called adeno-associated virus integration site 1 (*AAVS1*) locus, which maps to the *PPP1R12C* gene on chromosome 19 [72–74]. This site is characterized by an open chromatin structure and native insulators, which favor transcriptionally productive integrations [75,76]. Other GSHs commonly used in hPSCs include the human orthologue of the mouse *Rosa26* locus (*hROSA26*), mapping to the *THUMPD3-AS1* long non-coding RNA on chromosome 3 [77,78], and the citrate lyase beta-like (*CLYBL*) locus on chromosome 13 [79,80]. Comparative analyses indicate that both *hROSA26* and *CLYBL* can support even higher transgene expression in hPSCs and their derivatives compared to *AAVS1* [78,81].

Even though early studies suggested that these GSHs are robust targets for stable transgene expression in all hPSC-derived lineages, more detailed and extended investigations revealed a more complex picture. First, not all promoters are stably expressed from these loci. For instance, the EF-1alpha promoter, which, as mentioned above, is generally considered as "stem cell safe", does not support homogeneous transgene expression from neither the *AAVS1* nor the *hROSA26* locus [78]. Similarly, several cell type-specific promoters are not stably expressed from the *AAVS1* locus, including, paradoxically, the promoter of the pluripotency factor *POU5F1*/*OCT4* [24,82]. The most successfully used strategy to drive transgenesis from all three GSHs described above is using the CAG promoter (a hybrid sequence composed of the CMV early enhancer element, the promoter, first exon, and first intron of the chicken β-actin gene, and the splice acceptor of the rabbit β-globin gene) [74,78,81,83,84]. However, even the CAG promoter can be silenced in certain lineages, partially due to *de novo* DNA methylation of the transgene [85].

In all, while GSHs remain the best option for reproducible genetic engineering of hPSCs, investigators should carefully test novel promoters and challenging transgenes (i.e., long and/or non-eukaryotic sequences), for instance, by operationally linking the cassette to a fluorescent reporter to readily determine the homogeneity of expression in hPSCs and/or their derivatives (Fig. 1D). The addition of insulators flanking the transgenic cassette may be worthwhile. Last, but not least, it is recommended to exclude random integrations of the gene targeting vector, particularly if the goal is to generate an hPSC line stably expressing a transgenic cassette after differentiation. Indeed, additional off-target copies of the targeting vector can be silenced during differentiation due to chromatin remodeling. Random integrations of the targeting vector can be limited by minimizing the amount used for targeting and by relying on a promoter-less gene trap strategy to express a selectable marker to primarily select for the on-target modification.

Transient gene delivery

In some settings, the transient delivery of genetic material in hPSC models can be a useful alternative to the stable integration of nucleic acids. Adeno-associated viruses (AAVs) are popular non-integrating alternatives to lentiviruses, due to their low immunogenicity and ability to sustain long-term expression. Nevertheless, AAVs have a relatively small packaging limit, are laborious to produce, and, most crucially, can induce cell cycle arrest genes and apoptosis in hPSC, overall limiting their applicability to undifferentiated cells [49]. Despite these drawbacks, AAVs have been used to transiently modulate gene expression during hPSC differentiation, as well as in hPSC-derived cells such as hPSC-derived cardiomyocytes (hPSC-CMs; [49,50]). Of note, AAV efficiency is serotype dependent [86–88].

Non-viral transient nucleic acid delivery methods include the delivery of genetic material with chemically modified mRNAs (modRNA) or antisense oligonucleotides (ASO). modRNAs are stabilized to improve transgene expression and have been applied, for instance, for CRISPR editing of hPSCs through delivery of CRISPR/Cas9 components, leading to reduced off-target effects, low toxicity, and outstanding KO

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efficiency when compared to plasmid-based delivery [89]. This technology has also been applied to modify hPSC gene expression during differentiation [90]. ASOs are widely used to silence gene expression and have been also applied to hPSC [91]. ASOs target an mRNA by forming an mRNA-ASO hybrid that is then degraded by RNase H. ASOs must be stable as single-stranded oligonucleotides and find their target alone; therefore, chemical modifications could improve their stability and target efficiency. The use of ASOs in cultured cells has been limited due to their rapid degradation and stability when compared to other technologies such as siRNAs that do not need stabilization. However, when targeting organoids or tissues, stabilized ASOs have been a preferred choice over siRNA due to their ability to penetrate deeper and homogeneously [92–94]. ASOs have been rarely employed in undifferentiated hPSC, while they have been successfully used in hPSC derivatives [92,93,95–98] to test ASO-based therapies currently in clinical use.

Methods to induce gene loss-of-function

Reducing or ablating expression of a particular gene is not only the oldest trick in the functional genetics book but also an important tool to engineer cell function. Accordingly, loss-of-function (LoF) experiments have been performed since the discovery of hPSCs. At first RNA interference (RNAi) played a major role, but more recently a variety of CRISPR/Cas9-based methods have taken center stage. In this section, we describe the main methods to knockdown (KD) or knockout (KO) genes in hPSC models, with a particular focus on inducible LoF approaches that are ideal to study not only the pluripotent state but also hPSC-derived cells. We highlight the pros and cons of each method, which should be assessed before choosing the right tool for the job at hand (Fig. 2 and Table 1).

RNA interference

Some 25 years after its discovery [99], RNAi remains a key post-translational silencing technique. Three main types of RNAi molecules can be used in hPSCs, each mimicking the products of microRNA (miRNA) biogenesis: artificial miRNA (amiRNAs), usually encoded within mRNAs by RNA Pol II, to recapitulate pri-miRNAs; short hairpin RNAs (shRNAs), encoded by RNA Pol III as short RNAs that imitate Drosha-cleaved pre-miRNA; and short interfering RNAs (siRNA), double-stranded RNAs mimicking Dicer-processed miRNA duplexes, ready for loading by the RISC complex (reviewed in [100]). Transfected or electroporated siRNAs can be quite effective in hPSCs [35]. Still, as they are only transiently active, they are not suitable to study long-term LoF and/or hPSC-derived cells (unless these are themselves amenable to efficient siRNA delivery). First-generation amiRNAs (based on miR-30 design) and shRNAs have been extensively used in hPSC models (Table 1).

Short hairpin RNAs or amiRNAs can be delivered and expressed in hPSCs *via* plasmids [35,101], lentiviruses [102], transposons [51], and genome editing [103,104]. To allow temporal dissection of gene function and study of factors important for pluripotency, conditional shRNAs can be expressed using the Tet de-repressible system [105]. This relies on a modified H1 promoter silenced by the Tet-sensitive repressor protein (TetR) in the absence of Tet. The method can be implemented in hPSCs using lentiviruses [106] or by stable plasmid transfection [41]. Building upon this foundation, we previously developed the optimized inducible knockdown (OPTiKD) system, which combines a codon-optimized TetR with an all-in-one gene editing step in the *AAVS1* GSH to achieve reproducible, leak-proof, potent, homogeneous, and reversible shRNA expression in hPSCs (Fig. 2A; [78]). OPTiKD remains functional after differentiation into over a dozen hPSC-derived cell types from all three germ layers. The approach supports the expression of multiple shRNAs [107], and does not require clonal isolation of hPSCs [33].

RNAi methods, however, do not come without limitations. In particular, RNAi is characterized by poorly predictable off-target effects that differ from cell type to cell type [108,109]. Thus, the use of more than one shRNA against a given gene is key to confirm experimental observations. RNAi may not efficiently silence long non-coding RNAs (lncRNAs; [110]). Moreover, RNAi does not fully ablate gene expression [111], which can mask phenotypes for genes expressed at high levels and/or able to elicit their activity even in a haploinsufficient condition. Finally, the expression of shRNAs as a single copy, as for the OPTiKD approach, is even more sensitive to differences in shRNA potency that are still difficult to predict. Thus, it is important to test multiple shRNAs to identify those able to substantially downregulate protein expression (i.e., more than 70–80% KD). While there are publicly available genome-wide shRNA designs that can be consulted, such as the commonly used TRC library, these are not always effective. For instance, TRC shRNAs have limited efficacy under dose-limited conditions relevant for single-copy screens, and are not processed by DICER1 in the predicted manner [112]. Specifically, using this design DICER1

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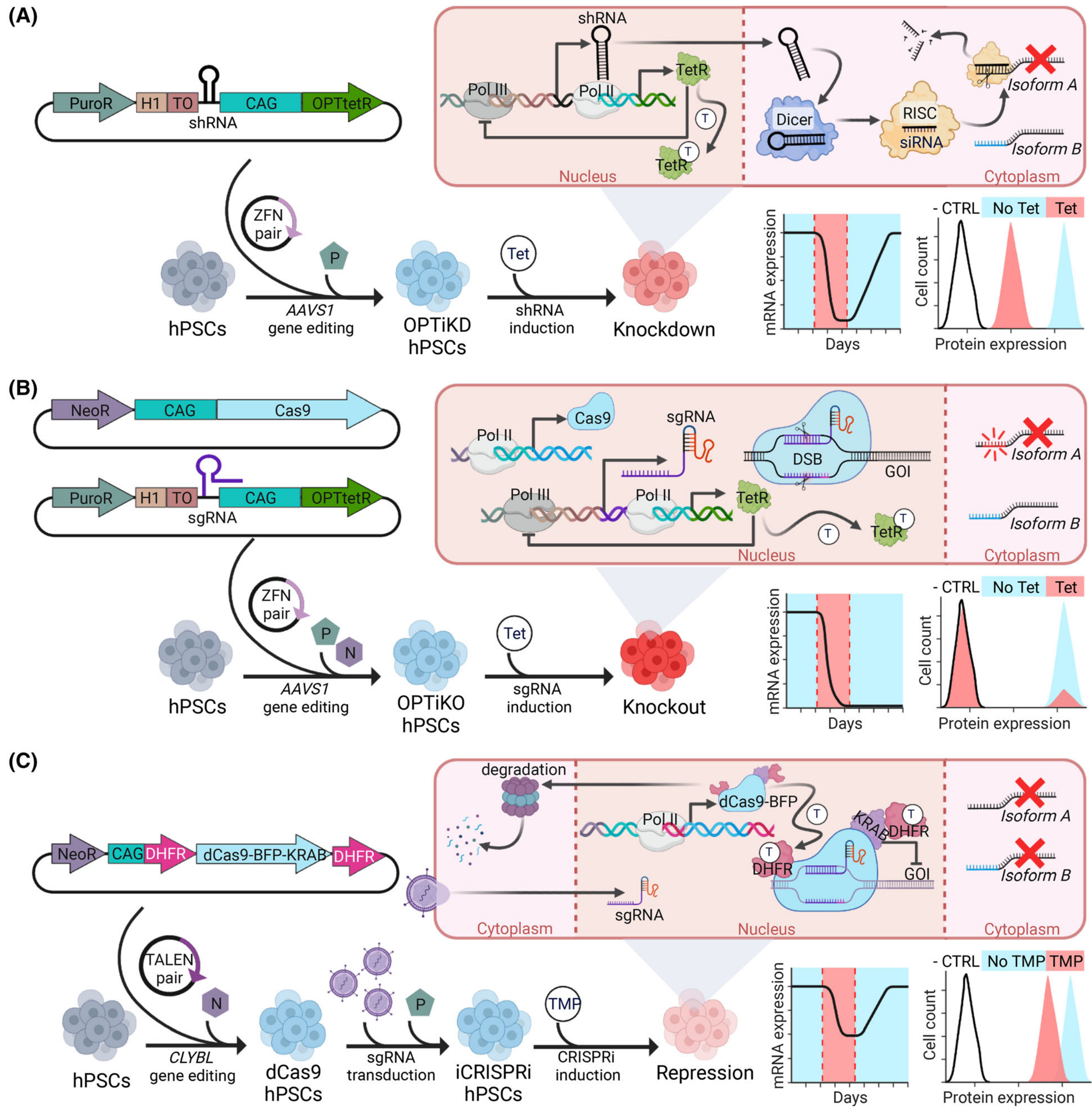

Fig. 2. Conditional loss-of-function in hPSC models. Exemplary methods for inducible RNAi, CRISPRn, and CRISPRi contrasted by genome-editing strategy, mode of action, and representative activity (inducibility, reversibility, and LoF strength and homogeneity). (A) The OPTiKD system is based on *AAVS1* genome editing with an all-in-one Tet-inducible shRNA cassette controlled by a codon-optimized tetR (OPTtetR/TetR; [78]). Tet treatment post-transcriptionally silences specific mRNA isoforms using the RNAi machinery, leading to rapid, reversible, homogeneous, and potent KD. H1: Pol III promoter; TO: tetracycline operon; P: Puromycin. (B) The OPTiKO relies on biallelic *AAVS1* genome editing with an all-in-one Tet-inducible sgRNA cassette and Cas9 [78]. Tet treatment leads to NHEJ-mediated KO (potentially of specific isoforms); KO can be rapid but usually not homogeneous and irreversible. N: Neomycin. (C) An inducible CRISPRi approach leverages on sequential *CLYBL* genome editing with a TMP-stabilizable CRISPRi effector and lentiviral transduction of sgRNA [139]. TMP treatment promotes epigenetic silencing of all mRNA isoforms encoded by a target locus, leading to rapid, reversible, and homogeneous KD.

appears to cleave 2–3 base pairs downstream of the intended cut site, resulting in shortened mature small RNA species with random 5′ ends, some of which cannot be effectively loaded into AGO2 [113]. This mis-cleavage might reflect incomplete fulfilling of sequence requirements for accurate DICER1 processing [114].

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Table 1. Exemplary methods for stable and conditional loss- and gain-of-function in hPSC models.

Approach	Effector	Delivery	On?	Off?	hPSC-lineages	Ref
Loss of function						
RNAi	CMV_amiRNA	Plasmid	No	No	Embryoid bodies	[98]
RNAi	CAG_amiRNA	Lentivirus	No	No	Hepatoblasts	[99]
RNAi	CA_2x-amiRNA	Gene editing (*AAVS1*)	No	No	Myeloid & erythroid cells; megakaryocytes	[100]
RNAi	TRE_amiRNA & TetON	Transposon	Dox	Yes	Embryoid bodies	[51]
RNAi	H1_shRNA	Plasmid	No	No	Embryoid bodies	[35]
RNAi	U6_shRNA	Gene editing (*AAVS1*)	No	No	Endoderm	[101]
RNAi	H1-TO_shRNA & CAG_TetR	Plasmid	Dox	Yes	N.D.	[41]
RNAi	H1-TO_shRNA & EF1- alpha_TetR	Lentivirus	Dox	Yes	Cardiomyocyte progenitors	[103]
RNAi (OPTiKD)	H1-TO_shRNA & CAG_OPTtetR	Gene editing (*AAVS1*)	Tet	Yes	12 cell types from all three germ layers	[78]
CRISPRn	CMV_Cas9 + U6_sgRNA	Plasmid	No	No	Endoderm and embryoid bodies	[101]
CRISPRn & recombination	CAG_Cas9-Cre & U6_sgRNA + CAG_Flpe-ERT2	Plasmid + gene editing (GOI & *AAVS1*)	OHT	No	Neuroepithelial	[114]
CRISPRn (iCRISPR)	TRE_Cas9 & CAG_M2rtTA + sgRNA	Gene editing (2 x *AAVS1*) + transfection	Dox	No	Pancreatic progenitors	[115]
CRISPRn	TRE3G_Cas9 & CAG_rtTA + sgRNA	Gene editing (*AAVS1*) + nucleofection	Dox	No	N.D.	[25]
CRISPRn (OPTiKO)	H1-TO_sgRNA & CAG_OPTtetR + CAG_Cas9	Gene editing (2 x *AAVS1*)	Tet	No	Hepatocytes, cardiomyocytes, neurons	[78]
CRISPRi	TRE_dCas9-KRAB & UBiC_rtTA3 + U6_sgRNA	Lentivirus	Dox	Yes	N.D.	[66]
CRISPRi	TRE3G_KRAB-dCas9-2A-mCherry & CAG_rtTA + sgRNA	Gene editing (*AAVS1*) + nucleofection	Dox	Yes	Cardiac progenitors, cardiomyocytes (weak)	[25]
CRISPRi	TRE3G_dCas9-HA-KRAB & CAG_rtTA + U6_sgRNA	Gene editing (*AAVS1*) + lentivirus	Dox	Yes	Hepatic endoderm	[138]
CRISPRi	TRE3G_KRAB-dCas9 & CAG_rtTA + U6_sgRNA	Gene editing (*AAVS1*) + transposon	Dox	Yes	N.D.	[54]
CRISPRi	CAG_dCas9-BFP-KRAB or CAG_ecDHFR-dCas9-BFP-KRAB-ecDHFR* + U6_sgRNA	Gene editing (*CLYBL*) + lentivirus	TMP*	Yes*	Neurons, astrocytes, microglia	[139–142]
CRIPRoff	CAG_DNMT3A-D3L-dCas9 + U6_sgRNAs	Plasmid + lentivirus	No	Yes	Neurons	[146]
Gain of function						
Transgenesis	CAG_GOI	Plasmid	No	No	Embryoid bodies	[35]
Transgenesis	EF1-alpha_GOI	Lentivirus	No	No	N.D.	[147]
Transgenesis	PGK_GOI + transposase	Transposon + plasmid	No	No	N.D.	[148]
Transgenesis	CAG_GOI	Gene editing (*AAVS1* or *hROSA26*)	No	No	12 cell types from all three germ layers	[74,78]

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Table 1. (Continued).

Approach	Effector	Delivery	On?	Off?	hPSC-lineages	Ref
Transgenesis recombination	CAG_CreERT2 + CAG_floxed-GOI	Plasmid	OHT	No	Embryoid bodies	[149]
Transgenesis	CMV-TetO_GOI & EF1-alpha_TetRnl2	Lentivirus	Dox	Yes	Mesenchymal and hematopoietic cells	[151]
Transgenesis	TRE_GOI & CAG_M2rtTA	Gene editing (2 x *AAVS1*)	Dox	Yes	Pancreatic progenitors	[115]
Transgenesis	CAG_TetOn3G & TRE3G_GOI (or M2rtTA & TRE)	Gene editing (*AAVS1*)	Dox	Yes	Teratomas (but inactive in hepatocytes)	[24,153]
Transgenesis (OPTiOX)	CAG_TetOn3G & TRE3G_GOI	Gene editing (*hROSA26* + *AAVS1*)	Dox	Yes	Neurons and pancreatic cells (but inactive in others)	[12,78]
CRISPRa	UBC_vp64-dCas9-vp64 (EF1-alpha_dCas9-VPR) + U6_sgRNA	Lentivirus	No	No	Neurons	[66,159,160]
CRISPRa	TRE_dCas9-VPR & CAG_M2rtTA + U6_sgRNA	Gene editing (2 x *AAVS1*) + plasmid	Dox	Yes	N.D.	[161]
CRISPRa	TRE3G_dCas9-VPR & CAG_rtTA + U6_sgRNA	Gene editing (*AAVS1*) + transposon	Dox	Yes	N.D.	[54]
CRISPRa	CAG_ecDHFR-dCas9-44xvp48-p65-HSF1 + U6_sgRNA	Gene editing (*CLYBL*) + lentivirus	TMP	Yes	Neurons and microglia	[140,142]

CRISPR nuclease

Human pluripotent stem cell KO lines can be generated using any programmable nuclease. However, CRISPR nuclease (CRISPRn) strategies have now virtually substituted ZFNs and TALENs, being easier to implement and not suffering from any specific additional limitation. Among CRISPR systems, Cas9 from *Streptococcus pyogenes* (SpCas9) has been widely utilized in hPSCs [61], though alternative Cas9 proteins (i.e., *Staphylococcus aureus* Cas9) can be useful in specific contexts (i.e., to target regions where no specific sgRNA for SpCas9 can be designed). Various CRISPR/Cas9 systems and their general principles have been excellently reviewed elsewhere [115].

Constitutive KO hPSCs can be obtained either by inducing a frameshift mutation through error-prone NHEJ repair of a single DSB, or by excising a larger region (i.e., by NHEJ repair of two distal DSBs or following HDR with a donor vector lacking the target region). Disruption of gene function can be achieved by targeting an exon common to all isoforms and encoding for an essential protein domain; alternatively, isoform-specific exons can be targeted to KO only a specific transcript. KO can be induced not only in coding genes but also for lncRNAs [104]. CRISPR/Cas9 can be transiently delivered using plasmids encoding both Cas9 and the sgRNA: operationally linking Cas9 expression with a selectable marker allows for enrichment of cells exposed to high levels of the nuclease [113]. Alternatively, Cas9 protein can be precomplexed to a synthetic sgRNA and delivered as a ribonucleoprotein complex [8], which is more efficiently delivered and has lower off-target effects, being more rapidly degraded.

Conditional KO hPSCs are advantageous for the same reasons described above for inducible RNAi; more so because full LoF can be even more detrimental to the pluripotent state. A recombination-based inducible KO approach relies on the insertion of flippase recognition target sites flanking an essential exon, whose excision can be triggered by a tamoxifen-inducible flippase previously knocked in the *AAVS1* GSH [114]. This elegant method is nevertheless relatively laborious, requiring at least two genome editing steps. An alternative approach called iCRISPR is based on Tet-On technology, whereby both a second-generation reverse tetracycline-controlled transactivator (rtTA) and a Dox-inducible Cas9 are each knocked into one of the alleles of the *AAVS1* GSH [115]. Transient transfection of sgRNAs combined with Dox treatment allows even multiplexed inducible gene KO. A related approach delivers similar transgenes, except for a third-generation rtTA, with an all-in-one

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cassette in the *AAVS1* locus [25], simplifying the genome editing. Both of these approaches, however, rely on the TRE promoter to drive Cas9: in line with *in vivo* mouse experiments [116], such promoter is not stably expressed following hPSC differentiation in multiple lineages [24,25,78], limiting the functionality of the methods. We developed another strategy, optimized inducible knockout (OPTiKO), whereby the temporal control of KO is at the level of sgRNA expression [78,117]. OPTiKO relies on targeting each allele of the *AAVS1* locus with an all-in-one Tet-inducible sgRNA cassette and a constitutively expressed Cas9, respectively (Fig. 2B). The approach is functional in neurons, cardiomyocytes, and pancreatic cells. Notably, however, the system is extremely sensitive to the potency of the sgRNA, which requires time-consuming optimization of sgRNA copy number and/or inducible promoter. When properly calibrated, OPTiKO allows leak-proof yet rapid induction of gene KO, even for essential pluripotency genes such as *POU5F1*/*OCT4* [118].

The potential off-target activity of Cas9 is well known [119]. However, whole-genome sequencing studies of hPSCs found that CRISPR/Cas9 and TALEN technologies exhibit minimal off-target mutations, and the incidence of mutations attributed to nucleases is no greater than those accumulated through routine hPSC culture [120–122]. Off-target activity is also predictable to a high degree of accuracy [123], and can be minimized by choosing highly selective sgRNAs [124]. Additionally, sgRNA modifications at the 5′ end can further reduce off-targets [125], and have even been reported to reduce p53 activity in hPSCs [126]. For specific applications, particularly those conducive to clinical trials, there are several Cas9 variants engineered to enhance their specificity, usually at the expense of lower efficacy [127]. For instance, both eSpCas9 and SpCas9-HF1 have been successfully used for mutation corrections [128,129] or disease modeling [130]. Alternatively, dual nickase strategies, which rely on a Cas9 nickase (nCas9) mutant with an inactive nuclease domain, are highly specific as they require paired recruitment to cut opposite DNA strands [131].

Off-targets aside, CRISPRn approaches carry their own set of concerns. First, the sensitivity of hPSCs to p53 activation clashes with methods that are intrinsically genotoxic. Secondly, inducible CRISPR/Cas9 approaches generate a mixed cell population carrying different mutations, including some that do not induce loss of gene function because they are in frame. Third, these methods are not reversible, preventing the study of gene function in a stage-specific manner. Finally, inactivating mutations can lead to genetic compensation [132,133] and mRNA misregulation [134] that can mask LoF phenotypes.

CRISPR interference

Catalytically inactive variants of Cas9 (dCas9, containing mutations in both nuclease domains) can suppress gene expression through simple steric interference with DNA polymerase [135] or by recruiting transcriptional repressors through direct protein fusion (i.e., dCas9-KRAB and dCas9-KRAB-MeCP2; [64,136]). Such an approach, which is usually referred to as CRISPR interference (CRISPRi), has limited off-target effects, is not genotoxic, is potentially reversible, and can target both coding and non-coding genes [137].

Conditional CRISPRi in hPSCs can be achieved using an all-in-one lentivirus encoding Dox-inducible dCas9-KRAB and a constitutive sgRNA [66], but the approach is subject to lentiviral silencing and hence results in mosaic activity. An alternative strategy based on genome editing of the *AAVS1* locus with KRAB-dCas9 allows both constitutive and conditional, Dox-inducible silencing [25]. The method allows for homogeneous, rapid, dose-dependent, and reversible LoF in hPSCs. In this approach, sgRNAs are delivered either by transient transfection or by random integration of a plasmid. Alternatively, similar strategies can be implemented whereby sgRNAs are delivered using lentivirus [138] or PB transposons [54]. Unfortunately, similar to the iCRISPR method described in the previous section, all of these approaches rely on the TRE promoter, which limits the applicability of the method to hPSC derivatives (i.e., poor activity in hPSC-CMs [25]).

A TRE-independent approach for conditional CRISPRi relies on a dCas9-BFP-KRAB tagged with two copies of the dihydrofolate reductase (DHFR) degron at the N and C terminus [139]. The transgene is constitutively expressed from the *CLYBL* locus, but is unstable in the absence of the small molecule trimethoprim (TMP), which can be added to trigger CRISPRi activity in cells whereby sgRNAs have been delivered using lentivirus (Fig. 2C). Alas, the method leads to less efficient gene silencing compared to a constitutive CRISPRi approach based on the same strategy except for the lack of degrons [139]. Both of these strategies are not only functional in ectodermal lineages such as neurons and astrocytes but also in some mesodermal lineages such as microglia [139–142]. Implementation of the constitutive CRISPRi strategy in hPSC-CMs, however, has been only moderately effective, possibly due to dCas9-BFP-KRAB silencing during differentiation [143].

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CRISPRi is a powerful approach that overcomes some key limitations of CRISPRn. However, because CRISPRi blocks transcription at promoter sites, it does not allow selective targeting of splicing isoforms derived from the same locus, nor of non-coding RNAs embedded in the introns of coding transcripts. Moreover, despite advancement in prediction tools, designing an optimal sgRNA for CRISPRi remains a matter of trial and error; with potentially more than one sgRNA required to induce complete KD [144]. Finally, CRISPRi relies on non-endogenous, high-molecular-weight dCas9 fusion proteins that are not easily delivered to hPSCs, may be poorly expressed (due to silencing and/or truncation during translation), and could be toxic when expressed at high levels and/or for prolonged periods.

CRISPR silencing

While CRISPRi is generally reversible, a specialized type of CRISPR-based epigenetic rewiring has been developed to induce inheritable gene silencing. The approach combines KRAB-dependent histone-based silencing with DNMT3A- and DNMT3L-dependent DNA-methylation, and was first established using TALE technology [145]. Refinements in protein engineering led to the development of a dCas9-based fusion protein containing all relevant enzymatic domains: CRISPRoff [146]. CRISPRoff silencing is maintained for more than 450 cell divisions. An opposite method, CRISPRon, can reverse the effects by using dCas9-TET1 to remove DNA methylation and by recruiting additional transcriptional activators through an RNA aptamer system. CRISPRoff has been applied to hPSCs through transient transfection, leading to stable silencing after 1 month of culture as well as after neuronal differentiation [146]. Alas, methods to perform conditional CRISPR silencing and, possibly, reactivation have not been yet reported.

Methods to induce gene gain-of-function

Amplifying gene function is another essential tool in functional genomics. Overexpression of genes not normally active in hPSCs or in hPSC derivatives has also emerged as a powerful approach to “forward program” stem or progenitor cells into mature lineages [11,12]. On the flip side of the coin, however, these findings highlighted the importance of conditional GoF methods, as overexpression of many genes can alter the delicate balance of the pluripotent state, leading to hPSC differentiation. In the following sections, we describe the main methods that have been developed to achieve this challenging task, either *via* conventional transgenesis or CRISPR-based systems, highlighting the pros and cons of each (Fig. 3; Table 1).

Transgenesis

Engineering hPSCs to express exogenous open reading frames (ORFs) remains a powerful approach. Constitutively expressed cDNAs driven by strong “stem cell-safe” promoters such as CAG or EF-1alpha can be delivered using plasmids [35], lentiviruses [147], transposons [148], or genome editing [74], but selective pressure for cells whereby the pluripotent state has not been disrupted can lead to inefficient results, particularly for genes involved in cell fate specification.

Conditional transgenesis can be achieved using a tamoxifen-inducible Cre recombinase system [149]. The approach relies on fusion of Cre with two copies of the estrogen receptor (ER), which retain Cre in the cytoplasm in normal conditions. Upon addition of tamoxifen or hydroxytamoxifen (OHT), Cre translocates to the nucleus where it excises a loxP-flanked cassette, leading to the reconstitution of an active ORF for a gene of interest (GOI; Fig. 3A). In its original iteration, the approach was based on plasmid delivery of all elements of the system.

The most popular approach for conditional transgenesis in hPSCs has been the Tet-On system. Lentiviral delivery of Dox-inducible transgenes can be effective [150], particularly if using constructs carrying insulators to minimize silencing [151]. Nevertheless, GSH integration of the two elements of the Tet-On system, rtTA and the Dox-inducible gene, provides more consistent results. This can be achieved by simultaneously targeting the two alleles of the *AAVS1* locus with one transgene each [115], by targeting a single allele with “all-in-one” strategy [152,153], or by targeting each element in a different GSH [12]. We pursued the last strategy to bypass limitations of all-in-one Tet-On designs [154], and to maximize copy number of each transgene. The resulting optimized inducible overexpression (OPTiOX) utilizes the *hROSA26* locus to constitutively express two copies of third-generation rtTA, and the *AAVS1* locus to express two copies of a TRE-driven Dox-inducible cassette (Fig. 3B; [12]). OPTiOX supports exceptionally strong, homogeneous, rapid, and reversible transgene overexpression, which can be leveraged, for instance, to maximize the efficiency of hPSC forward programming into a variety of lineages including excitatory neurons, skeletal muscle, oligodendrocytes, astrocytes, hepatocytes, and microglia [12,155–158]. The major limitation of both OPTiOX and other TRE-based systems is the

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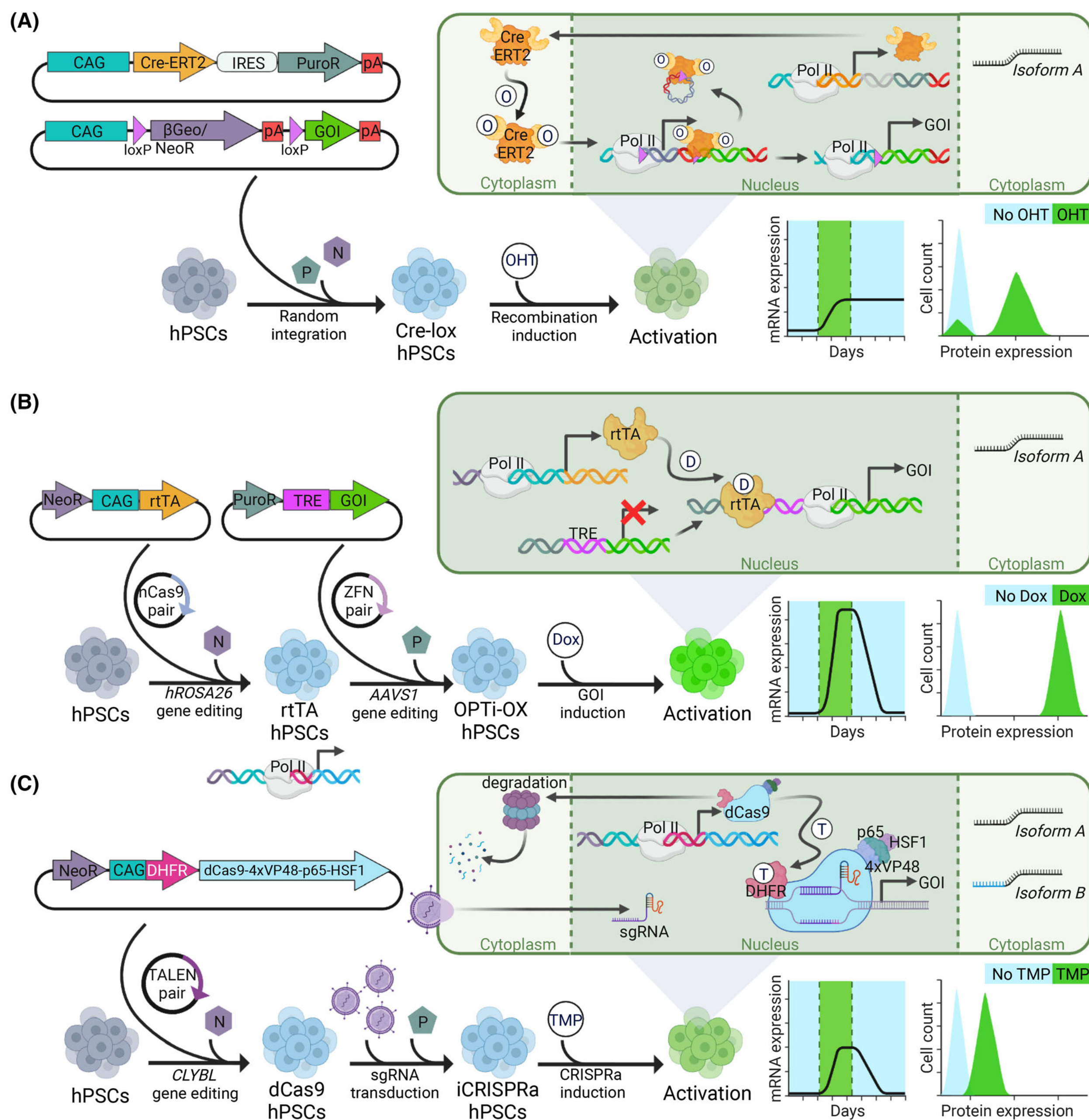

Fig. 3. Conditional gain-of-function in hPSC models. Exemplary methods for inducible recombination, transgenesis, and CRISPRa contrasted by genome-editing strategy, mode of action, and representative activity (inducibility, reversibility, and GoF strength and homogeneity). (A) A Cre-ERT2-loxP system based on random integration of two plasmids irreversibly activate a GOI following OHT treatment, leading to strong GoF in a majority of cells [149]. βGeo/NeoR: β-galactosidase and aminoglycoside 3′-phosphotransferase fusion; pA: polyadenylation signal. (B) The OPTiOX system relies on dual GSH editing of *hROSA26* and *AAVS1* with rtTA a Dox-responsive GOI, respectively [12]. Dox treatment leads to homogeneous, reversible, and ultra potent GoF, but the method is not fully active in certain hPSC-derived lineages (not shown; [78]). N: Neomycin. (C) An inducible CRISPRa approach leverages on sequential *CLYBL* genome editing with a TMP-stabilizable CRISPRa effector and lentiviral transduction of sgRNA [139]. TMP treatment promotes epigenetic activation of all mRNA isoforms encoded by a target locus, leading to rapid, reversible, and homogeneous GoF.

aforementioned inactivation of this promoter after hPSC differentiation: by testing six lineages we demonstrated poor activity of OPTiOX in hPSC-derived cardiomyocytes, smooth muscle cells, and hepatocytes; only neurons and pancreatic cells showed potent transgene inducibility [78].

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CRISPR activation

dCas9 fusion proteins can be leveraged to recruit transcriptional activator domains at promoter and/or enhancer sites, a strategy called CRISPR activation (CRISPRa; [65]). A lentiviral construct encoding dCas9 fused to VP64—4 copies of the Herpes simplex virus virion protein 16 (VP16)—leads to mosaic CRISPRa in hPSCs co-transduced with one or more sgRNAs against a target locus, the latter leading to synergistic effects [66]. A similar strategy leverages lentiviral dCas9 fused to two copies of VP64 at the N and C terminus [159]. A second-generation lentiviral CRISPRa effector constituted by dCas9 fused to VP64-p65-Rta (VPR) leads to strong transcriptional activation [160], which can, for instance, support forward programming of hPSCs in neurons following co-transduction of a pool of 30 sgRNAs directed against master TFs *NGN2* or *NEUROD1*. To improve the homogeneity of CRISPRa and allow conditional applications, Dox-inducible dCas9-VPR hPSCs can be generated by either targeting two alleles of the *AAVS1* locus [161] or using an all-in-one cassette [54]. sgRNA are delivered *via* plasmid or PB transposon, followed by Dox-induction of CRISPRa. Both methods are, however, subject to the limitations linked to the TRE promoter discussed above.

Comparative analyses of several CRISPRa effectors in other cell types indicate CRISPR synergistic activation mediator (SAM) as the most effective approach for a diversity of loci [162]. This method combines dCas9-VP64 with a modified sgRNA containing MS2 aptamers, which can recruit a fusion protein between the viral RNA binding protein MS2 capsid protein (MCP) and the p65-HSF1 (PH) transcriptional activation domains [163]. hPSCs stably transduced with the CRISPR-SAM system components have been generated but not characterized for their ability to mediate CRISPRa before or after differentiation [164]. The CRISPRon system relies on a similar approach based on using a modified sgRNA with MS2 hairpins to recruit MCP-VPR to sites bound by dCas9-TET1, but has not yet been tested in hPSCs [146].

An alternative to Dox-inducible conditional CRISPRa relies on the DHFR-TMP degron system (Fig. 3C). In this case, the *CLYBL* locus is edited with dCas9 fused to DHFR at its N terminus and to a multidomain activator at its C terminus, composed of four copies of VP48, p65, and HSF1 [140]. As for the related TMP-inducible CRISPRi approach, the method was validated to function not only in hPSCs but also in differentiated cells such as neurons and microglia [140,142].

CRISPRa is attractive compared to conventional transgenesis, as it does not require time-consuming and complex cloning of cDNAs. It is also in principle scalable to multiple genes and reversible. Nevertheless, it suffers from similar limitations to those described for CRISPRi regarding the inability to overexpress specific gene isoforms, the requirement for extensive optimization of multiplexed sgRNA design for maximal results, and issues associated with large dCas9 fusion proteins overexpression. Last but not least, the level of overexpression that can be achieved with CRISPRa is generally lower than conventional transgenesis, and is locus dependent [165,166].

Methods to induce gene change-of-function

Altering gene function with a knockin (KI) is the third major way to understand gene function, for instance, by recapitulating patient-derived mutations in healthy hiPSCs or by correcting them in patient-derived hiPSCs to generate isogenic controls. Besides point mutations, genes can be altered by introducing tags (i.e., fluorescent reporters) or through even more complex rearrangements. Established yet cumbersome approaches for KI rely on HR (Fig. 4), while recent developments in CRISPR base editing (BE) and prime editing (PE) are opening the door to more systematic KI studies (Fig. 5).

Homologous recombination

Precision editing can be achieved through the endogenous HDR machinery by supplying the cells with a plasmid or a single-stranded oligodeoxynucleotide (ssODN) template, carrying the mutation of interest, while simultaneously inducing a DSB at the target locus using a programmable nuclease, usually CRISPR/Cas9 [167]. As already mentioned, however, HDR is normally quite inefficient in hPSC. As such, precision genome editing is still time consuming and challenging, with an overall efficiency of around 1% [168]. Various tricks have been developed to ease the burden of screening hundreds of clones.

Positive selection

Reporter systems or selectable markers operationally linked to the HR template have been widely utilized to simplify the identification of genome-edited cells [77]. Selection based on antibiotic resistance is efficient, but it needs to be carefully planned as excessive selective pressure may lead to indels and off-target integrations [169]. An antibiotic kill curve should be performed to identify the lowest concentration in which non-

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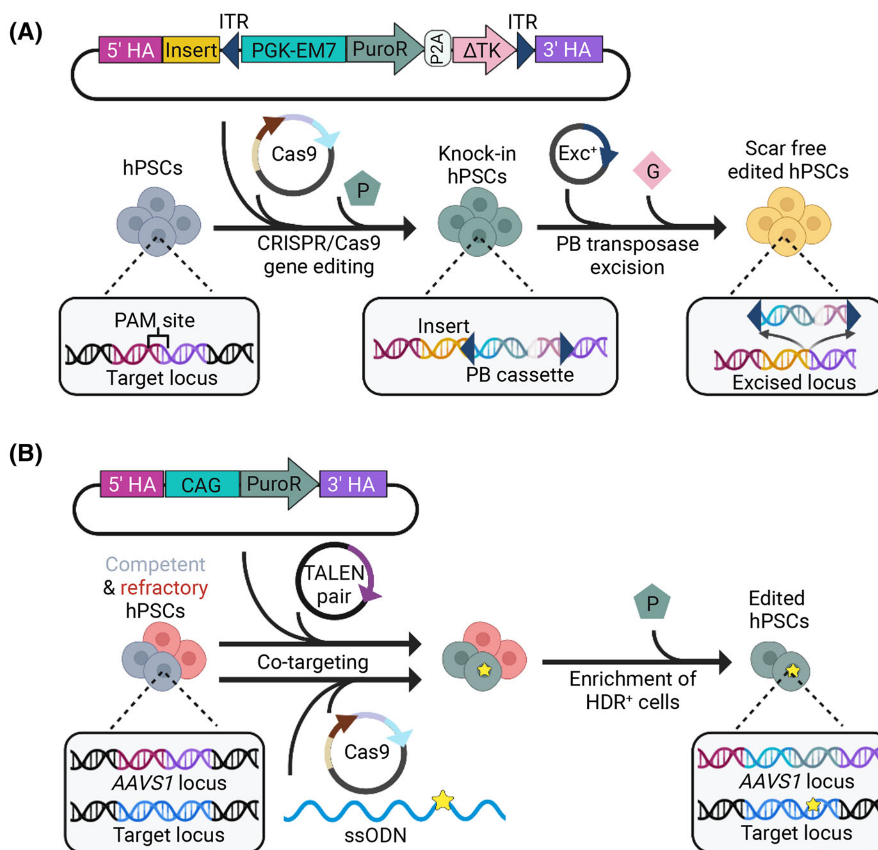

Fig. 4. Change of function by homologous recombination in hPSCs. Exemplary methods to KI large insertions or point mutations by HR. (A) Two-step scarless KI of an insert facilitated by sequential insertion and removal of a PB encoding for dual positive and negative selectable markers [176]. 5′/3′ HA: 5′ and 3′ homology arms; PGK-EM7: constitutive hybrid Pol II promoter; P2A: self-cleaving viral peptide; ΔTK: truncated Herpes simplex virus thymidine kinase; PAM: protospacer adjacent motif; Exc^{+}: excision only transposase; G: Ganciclovir. (B) Co-targeting of a targeted mutation with a selectable passenger modification (i.e., *AAVS1* genome editing) to enrich edited cells [184].

resistant cells are no longer viable [170]. If the editing efficiency is sufficiently high, antibiotic selection can be transient and based on a gene encoded outside the HR template, merely to enrich hPSCs that received the template [169]. Fluorescent reporters are valuable alternatives for transient and/or stable selection [171].

Selectable markers can be expressed through exogenous promoters or can be operationally linked to the endogenous promoter of the gene subject to KI (i.e., gene trap). The latter is particularly useful to generate fluorescent reporter KI [171]. However, this strategy is challenging to apply to genes that are silent in hPSCs. One solution is to use a second selectable reporter driven by a dedicated reporter for the positive selection, which can then be removed (i.e., through Cre-loxP recombination of CRISPR/Cas9 excision; [172]). An alternative approach is to transiently activate the silent locus through CRISPRa, so as to enable a well-timed positive selection [173].

Negative selection

Inverting the logic described in the previous section, it is possible to generate an hPSC line expressing a marker within a GOI, and selecting cells that eliminated such marker after HR. The universal knockin system (UKiS) deploys this strategy by first generating a mono- or biallelic hPSC line expressing fluorescent markers, and then using FACS to select cells that undergo HR with a template carrying a desired mutation [174].

Scarless editing

There are cases that require leaving no genomic scar after KI, such as the correction of mutations in patient-derived hiPSCs to generate isogenic controls. Since both Cre-loxP recombination and CRISPR/Cas9 excision leave definite scars, they are not suitable for the task. Instead, it is possible to use the PB

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transposon for a slow yet effective two-step procedure that ultimately leaves no genomic scar (Fig. 4A; [175,176]). In this approach a PB carrying a selectable marker (i.e., puromycin N-acetyl-transferase) is inserted as part of a larger transgenic cassette to allow positive selection of HR. The transposon is then removed by an excision-only transposase, followed by negative selection of hPSCs still carrying the transposon through a second marker contained within (i.e., the herpes simplex virus thymidine kinase gene, which sensitizes cells to the pro-drug Ganciclovir or its analog Fialuridine [177]). When the transposon is initially inserted in a region containing a TTAA, this procedure leaves no detectable genomic scar. FACE (FACS-assisted CRISPR/Cas9 editing) is a similar approach relying on HR with templates containing pairs of mutations and PB-excisable fluorescent proteins, which are used to select cells carrying precise genotypes and facilitate the isolation of compound heterozygous or homozygous mutants [178].

Of note, studies in mouse ESCs indicate that PB transposition can be inhibited by DNA methylation of the transposon [179]. Moreover, silencing of the negative selection marker can prevent its successful utilization to isolate hPSCs lacking the transposon. Therefore, it is advisable to operationally link the positive and negative selection (i.e., expressing the two genes in a polycistronic construct) in order to maintain the positive selective pressure until excision is triggered [176]. An alternative option for scarless genome editing is to avoid selectable markers altogether and brute force screen many clones, ideally paired to some prior selection of clonal pools enriched for the desired edit [180].

Co-selection

Compared to using plasmid templates, genome editing using ssODN reduces costs, cloning complexity, and delivery [181,182]. This approach does not allow the use of selective markers to directly enrich genome-edited cells, but it is possible to do so indirectly through co-selection. In this general strategy, cells are co-targeted with the construct for the mutation of interest and an independent selectable marker [183,184]. By co-selecting for a passenger HDR-based edit, this approach enriches the primary editing ~-10-fold or more. This is thought to arise from the selection of cells where the genome editing reagents are efficiently delivered and that are competent for HDR (i.e., they are in the correct phase of the cell cycle and they strongly express the HDR machinery). One possibility that has been implemented in hPSCs is to co-select based on editing a GSH such as the *AAVS1*, as the resulting mutation is expected to have no substantial effects on hPSCs (Fig. 4B; [184]). However, the outcome of co-targeting varies from cell line to cell line, and depends on the chromatin state of the locus of interest.

CRISPR systems

All of the methods described in the previous section share the downside of being genotoxic, which as discussed earlier is a major limitation of CRISPRn approaches in hPSCs. Programmable base editors and prime editors that mutate the DNA without inducing a DSB offer a promising alternative (Fig. 5; reviewed in [112]).

Base editing

CRISPR BE relies on dCas9 fusion proteins with nucleotide mutating enzymes to create cytosine base editors (CBEs, which catalyze the conversion of C-G base pairs to T-A) and adenine base editors (ABEs, which catalyze A-T to G-C conversions [185,186]). Collectively, CBEs and ABEs can mediate all four possible transition mutations (C → T, A → G, T → C, G → A). Base editors can be delivered to hPSCs as mRNA [187] or plasmid [188]. To increase the efficiency of BE, it can be advantageous to use a plasmid which contains an eukaryotic origin of replication supporting episomal expression for several cell generations [189]. Alternatively, base editors can be introduced as a Dox-inducible system in the *AAVS1* locus (Fig. 5; [190]). Another approach that can enrich base-edited hPSCs is the use of a transient reporter for editing enrichment (TREE): a fluorescent marker that is co-base-edited with the modification of interest [191].

Prime editing

CRISPR/Cas9 PE exploits Cas9 nickase fused with an engineered reverse transcriptase, and is guided by a prime-editing guide RNA (pegRNA), which is composed of both the sgRNA and the template encoding the mutation to be introduced [192]. Through DNA nicking, pegRNA hybridization and reverse transcription, flap cleavage, ligation, and mismatch repair, PE can introduce point mutations or even small indels. PE components can be delivered in hPSCs as plasmid [193], RNP, or mRNA [194]. They have also been introduced through genome editing in the *AAVS1* locus [194], including as a Dox-inducible system (Fig. 5; [190]). PE efficiency in hPSCs can be improved by expressing the pegRNA as Pol II-

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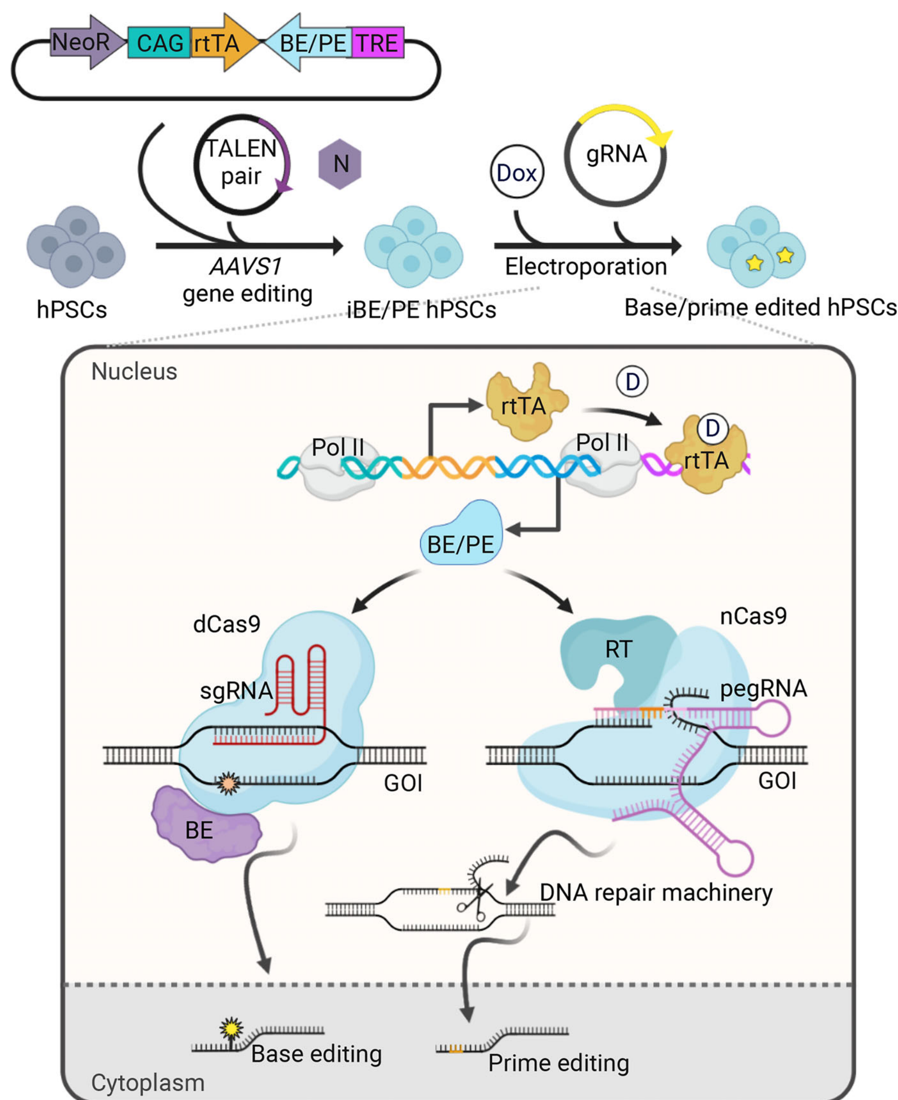

Fig. 5. Change of function by CRISPR in hPSCs. Exemplary approach to insert point mutations or small indels by CRISPR base editing or prime editing. Dox-inducible BE or PE hPSCs are obtained by *AAVS1* genome editing, exposed to Dox, and electroporated with a sgRNA or pegRNA to obtain base edited or prime edited cells [190].

driven intronic elements processed by the CRISPR-associated protein Csy4 [195].

While BE and PE are less efficient in hPSCs than in other cell types, they seem to be equally or even more effective than HR for most KI experiments. While BE and PE do not induce a DSB, they seem to nevertheless activate p53 in hPSCs: co-transfection of an episomal vector that expresses a dominant-negative version of p53 (p53DD) [67,194] or a p53DD modRNA [86] can transiently block p53 function and therefore reduce Cas9-induced toxicity in hPSC and increase the efficiency of CRISPR systems including BE and PE [194]. The Dox-inducible BE and PE approaches allow previously challenging conditional KI studies. Improvements in BE specificity are key to limit bystanding mutations, which represent the major limitation of this approach [196]. PE is less efficient than BE, but more specific [190]. While BE and PE are increasingly substituting HR for the introduction of point mutations in hPSCs, HR will likely continue to be essential to introduce large mutations currently not supported by PE.

High-throughput functional genomics

The rapid assessment of a large number of genotype–phenotype associations is an essential problem of

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modern genetics. While historical methods based on dropout and/or enrichment screening continue to play a major role, recently developed hypothesis-free approaches based on single-cell RNA sequencing (sc-RNA-seq) have taken center stage. Such methods have been particularly successful when coupled with CRISPR/Cas9 technologies (recently reviewed in [197]). In the following sections, we outline how the LoF, GoF, and CoF methods described above can be applied for functional genomic studies in hPSC models, with a particular focus on studies of development and disease mechanisms (Table 2). We then describe the essential analytical methods tailored for these studies.

Types of functional genomic screens

Functional screens can be performed either in an arrayed or pooled format (Fig. 6). Arrayed screens have generally lower throughput, and the phenotypic analysis can be laborious (i.e., *via* high content imaging, fluorescence, or luminescence (reviewed in [198]). Pooled screenings facilitate genome-scale analyses (reviewed in [199]). Dropout/enrichment screens are the most common and are usually read out in bulk by using NGS to compare the genomic distribution of perturbations between the starting and the final condition. This approach allows only the description of broad phenotypes, such as viability, proliferation, or differentiation. More complex questions about differentiation or subtler phenotypes can be tackled by using, for instance, reporter hPSC lines for FACS analyses [200]. Even more complex questions and/or hypothesis-free screens can be achieved by using sc-RNA-seq to identify the transcriptional phenotype relative to a perturbation. While this approach can also be implemented for RNAi screens [201], it has found its most popular application for CRISPR screens (reviewed in [202]). Briefly, single-cell CRISPR/Cas9 screens detect both single-cell transcriptomes and the associated CRISPR perturbation by either detecting the guide itself or a paired polyadenylated barcode. These methods include CRISPR-seq [203], Perturb-seq [204], Mosaic-seq [205], and CROP-seq [206].

Loss-of-function screens

Loss-of-function remains the most widely used approach for functional genomics: not only experimental and analytical methods are well established, but the interpretation of data is more straightforward. Compared to established cell lines, however, hPSC models pose important considerations to plan and execute a successful LoF screen.

RNA interference

Arrayed RNAi screens can be performed by reverse transfection of hPSCs with siRNA libraries targeting a gene panel [207] or even the whole genome [208]. While siRNAs are inherently transient, this approach can be successfully applied to also study some aspects of hPSC differentiation (Fig. 6A; [207]). Pooled RNAi screens in hPSCs generally rely on the use of lentiviruses, which can also be applied to hPSC-derived cells to bypass lentiviral silencing in the pluripotent state [209].

The design of RNAi screens is nowadays fast and straightforward, given the availability of genome-wide RNAi libraries [210]. However, the limitations in KD efficiency and off-target effects become even more problematic when increasing the throughput of the RNAi perturbations [105,211–213].

CRISPR nuclease

CRISPRn screens in hPSC models reported to date have investigated from 172 to all coding genes in a pooled format, mostly relying on bulk sequencing to detect changes in sgRNA abundance between experimental conditions (Table 2). Pooled CRISPRn screens can be performed by transducing hPSCs with a lentivirus encoding both Cas9 and a sgRNA library (i.e., to study survival and/or proliferation [214]). To increase efficiency of lentiviral packaging and transduction, hPSCs can be transduced with separate lentivirus encoding for Cas9 and the sgRNA lentiviral library: either at the same time—less efficient but safer—or sequentially—more efficient but subject to Cas9 silencing. sgRNA-transduced hPSCs can be differentiated and selected based on lineages (i.e., FACS for fluorescent reporter or antibody staining [200,215–217]) or subjected to selective pressures (i.e., treatment with chemotherapeutic [218]). Alternatively, transduction can be performed at a specific stage of hPSC differentiation (i.e., macrophage precursors [219]) and/or prior to challenge (i.e., Zika virus [220]). Of note, since CRISPRn-induced KO is permanent, lentiviral silencing on the long term is less problematic compared to shRNA which must be constantly expressed.

Genome-wide screenings require a relatively homogeneous and sufficiently large starting cell population to ensure coverage of all perturbations (~100–1000 cells per sgRNA); this can be challenging in hPSC-derived lineages due to inefficient, poorly scalable, and highly variable differentiation protocols. A strategy to overcome this limitation is to combine a GoF approach for efficient forward programming of hPSCs

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Table 2. Published loss-, gain- and change-of-function screens in hPSC models.

Method	Delivery	Strategy	Throughput	Lineage	Ref
Loss of function					
RNAi (shRNA)	Lentivirus	Survival/dropout by TRA-1-81 staining FACS and gDNA-seq	~ 600 000 shRNAs (30 shRNAs per gene, targeting 19 527 genes)	hPSC-reprogrammed fibroblasts	[153]
RNAi (siRNA)	Reverse transfection	Arrayed screening by K18 staining	384 siRNA (4 siRNAs per gene)	hPSC-epithelial cells	[207]
RNAi (siRNA)	Reverse transfection	Arrayed GFP fluorescence with a *POU5F1* reporter	21 121 genes on 67 384-well plates	hPSCs	[208]
RNAi (shRNA)	Lentivirus	Survival/dropout by gDNA-seq at different time points	538 genes ~ 12 shRNA per gene (6482 shRNAs)	hPSC-HSCs and primary NSCs	[209]
CRISPRn	Lentivirus	Survival/dropout by FACS and gDNA-seq	18 080 genes with 64 751 sgRNA	hPSC- hepatocytes	[200]
CRISPRn	Lentivirus	Survival/dropout by gDNA-seq	~ 30 sgRNA per gene 5191 intergenic- and protein coding-targeted sgRNAs	hPSC-neurons	[220]
CRISPRn	Lentivirus	Survival/dropout by gDNA-seq	Brunello library (1841 sgRNA)	hPSC-cardiomyocytes	[218]
CRISPRn	Lentivirus	Survival/dropout by gDNA-seq	736 kinase	hPSC-cortical neurons	[225]
CRISPRn	Lentivirus (post diff)	Survival/dropout by GFP+ FACS and gDNA-seq	18 466 exons	Forward programmed neurons	[221]
CRISPRn	Lentivirus (post diff)	Survival/dropout by gDNA-seq and RNA-seq	26 306 sgRNAs targeting 4401 genes and 1000 controls	Forward programmed hPSC-neurons	[222]
CRISPRn	Lentivirus	sgRNA direct capture by sc-RNA-seq	431 sgRNAs (104 genes)	hPSCs	[144]
CRISPRn	Lentivirus	Survival/dropout by FACS and gDNA-seq	123 411 sgRNAs (6 sgRNA per gene)	hPSC-neural progenitor cells	[215]
CRISPRn	Lentivirus	FACS Dlxi1/2b EGFP bulk NGS, bulk RNA-seq, and sc-RNA-seq	425 neurodevelopmental related genes 5 sgRNA per gene and 218 sgRNAs controls	hPSC-subpallium organoids and forebrain assembloids	[216]
CRISPRn	Lentivirus	Survival/dropout by FACS, bulk NGS, RNA-seq, and sc-RNA-seq	6000 genes	hPSC-cardiac mesoderm and progenitors	[217]
CRISPRn	Lentivirus	Survival/dropout by FACS and gDNA-seq	TKOv3 library 71 090 sgRNA	hPSC-macrophages	[219]
CRISPRn	Lentivirus	Survival/dropout by FACS and gDNA-seq	TKOv3 library 71 090 sgRNA	hPSCs	[223]
CRISPRn	Lentivirus	Survival/dropout with lineage barcoded sgRNA	172 microcephaly candidate genes	hPSC-cerebral organoids	[227]
CRISPRn	Lentivirus	Survival/dropout by FACS and gDNA-seq	120 000 sgRNA	hPSCs	[228]
CRISPRi	Lentivirus	Survival/dropout by gDNA-seq; polyA sgRNA; sc-RNA-seq	2131 genes (5 sgRNA per gene)	Forward programmed hPSC-neurons	[140]
CRISPRi	Lentivirus	Survival/dropout by gDNA-seq; polyA sgRNA; sc-RNA-seq	2325 genes (5 sgRNA per gene)	Forward programmed hPSC-microglia	[142]
CRISPRi	Lentivirus	Survival/dropout by gDNA-seq; polyA sgRNA; sc-RNA-seq	58 sgRNAs (27 genes)	Forward programmed hPSC-neurons	[139]
CRISPRi	Lentivirus	Barcoded sgRNA identified by sc-RNA-seq	200 000 sgRNA for 30 000 target genes coding and non-coding	hPSC-neural stem cells	[230]

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Table 2. (Continued).

Method	Delivery	Strategy	Throughput	Lineage	Ref
CRISPRi	Lentivirus	FACS and sc-RNA-seq with polyA sgRNA	5 sgRNA per gene (~ 4000 targets)	hPSC-derived astrocytes	[141]
CRISPRi	Lentivirus	Survival/dropout and gDNA-seq	18 000 genes with 3 sgRNA per gene	hPSCs	[229]
CRISPRi	Lentivirus	Survival/dropout by FACS and gDNA-seq and bulk RNA-seq	90 000 sgRNA	hPSCs	[224]
CRISPRi	Lentivirus	Barcoded sgRNA for sc-RNA-seq detection	160 sgRNAs	hPSC-endoderm	[138]
Gain of function					
Transgenesis	PB transposon	Arrayed screen by morphology & RNA-seq	714 ORFs	hPSCs	[233]
Transgenesis	Lentivirus	Barcoded ORF detected by sc-RNAseq	431 TF	hPSC	[234]
Transgenesis	PB transposon	Barcoded ORF detected by sc-RNAseq	1732 splice isoforms of 1564 TFs	hPSCs	[235]
Transgenesis	PB transposon	Barcoded vectors, barcode amplification by bulk NGS (Bar-seq)	948 ORFs	hPSCs	[165]
CRISPRa	Lentivirus	Survival/dropout by gDNA-seq; polyA sgRNA; sc-RNA-seq	2131 genes (5 sgRNAs per gene)	Forward programmed hPSC-neurons	[140]
CRISPRa	Lentivirus	Survival/dropout by gDNA-seq; polyA sgRNA; sc-RNA-seq	2325 genes (5 sgRNA per gene)	Forward programmed hPSC-microglia	[142]
CRISPRa	Lentivirus	FACS after differentiation and then enrichment/depletion, gDNA-seq and RNA-seq	CAS TF library 1496 genes (8435 sgRNA, ~ 5 sgRNA per gene and 100 sgRNA scramble)	hPSC-neurons	[159]
Change of function					
DICE	Phi31 and Bxb1	Clonal genotyping	120 *TNNT2* variants	hPSC-cardiomyocytes	[240]
CRaTER	Plasmid	gDNA-seq and clonal genotyping	Saturation mutagenesis of 5 aa in *MYH7*	hPSC-cardiomyocytes	[173]
STRAIGHT-IN	Bxb1 or φC3	Clonal genotyping	12 *KCNH2* variants	hiPSC-cardiomyocytes	[238]

into highly homogeneous cortical neurons (NGN2 OPTi-OX) and a constitutively expressed dCas9 genome edited into the *GAPDH* locus to ensure its stable expression following differentiation [221]. After Dox-induced forward programming, neurons can be transduced with genome-wide sgRNA libraries, and subjected to a combination of selective pressures (i.e., tunicamycin [222] or cold shock [221]) and FACS enrichment, and analyzed by bulk NGS.

Another possibility to achieve consistent Cas9 expression during pooled CRISPRn screen is *via* genome editing into the *AAVS1* locus of hPSCs [25]. Such an hiPSC line can then be transduced with a library of sgRNAs for 3′ direct-capture Perturb-seq, in which expressed sgRNAs are sequenced alongside single-cell transcriptomes [144]. Compared to other single cell screening approaches, this method requires a specialized sgRNA library whereby a capture sequence is inserted in the stem loop. Alternatively, for compatibility with conventional sgRNA libraries, sgRNAs could be captured at the 5′ end, though this approach was not demonstrated in hPSC models [144].

Conditional pooled CRISPRn screens enable the study of gene function at specific developmental stages. This can be performed by gene editing hPSCs with a Dox-inducible Cas9, which is then transduced with a sgRNA library [223,224]. This approach has been successful in cortical neurons [225] and developing kidney organoids [226]. These studies showed that the addition of an insulator prior to the TRE promoter of the Dox-inducible Cas9 hPSC line can reduce silencing in differentiated cells of this otherwise troublesome promoter. An alternative tamoxifen-inducible CRISPRn screening strategy relies on transduction with a lentiviral pool that besides the sgRNA library delivers Cre-ERT2, which can be activated by OHT at specific stages on

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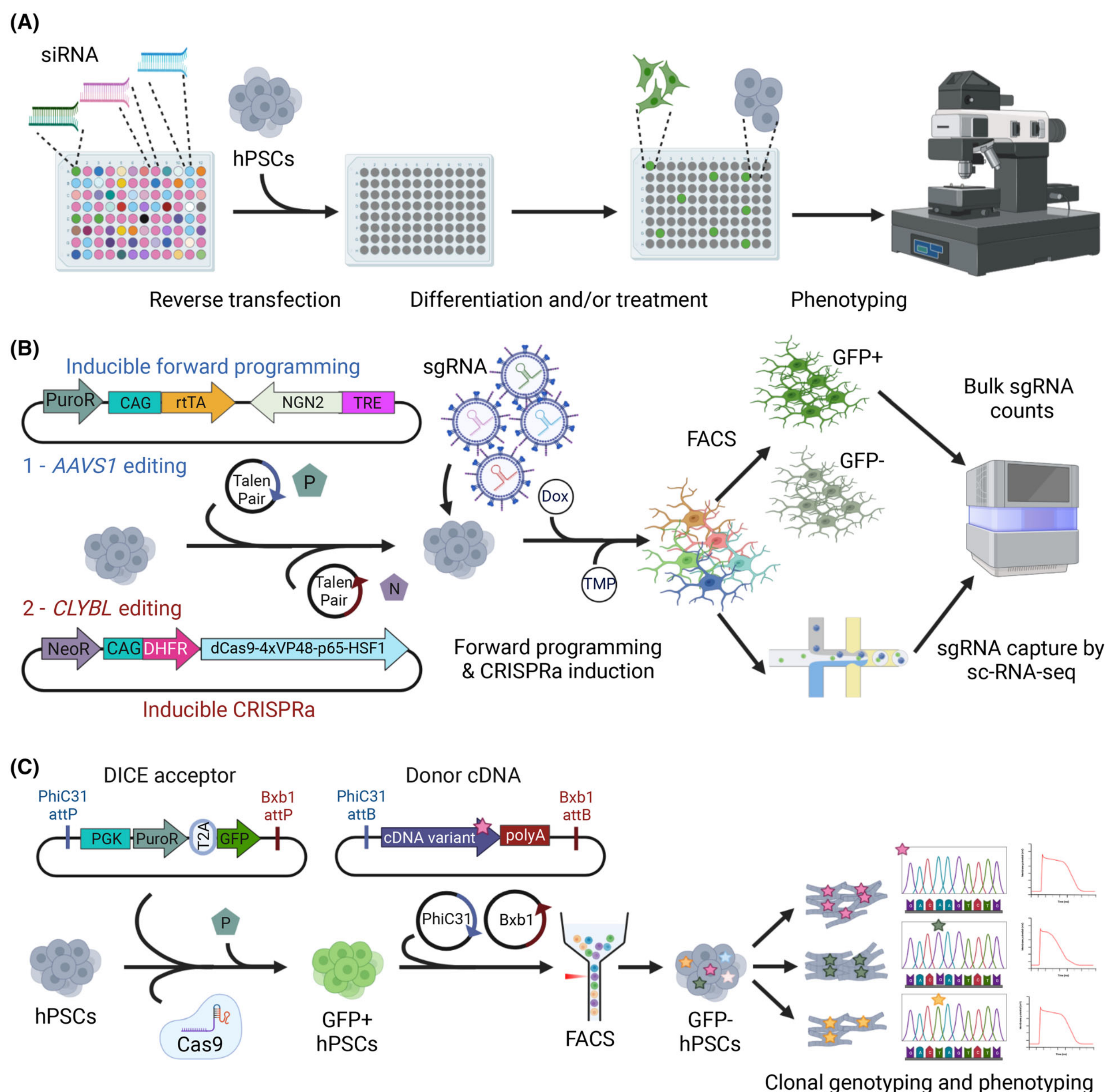

Fig. 6. High-throughput screening in hPSC models. Exemplary methods for LoF, GoF, and CoF screens in hPSC models using arrayed, pooled, single cell, and clonal strategies. (A) Arrayed LoF screen using RNAi spotted on multi wells and individually reverse transfected in hPSCs [208]. (B) Pooled and single cell GoF screen with inducible CRISPRa and a lentiviral sgRNA library (Fig. 3C), deployed in hPSCs previously engineered with Dox-inducible NGN2 to enable forward programming into neurons prior to CRISPRa induction [140]. (C) Clonal CoF screen using DICE [240], which creates a landing pad for dual integrase-mediated recombination of multiple gene variants enriched by negative FACS selection.

differentiation to activate an eSpCas9 gene (similar to the strategy described in Fig. 3A). This approach has been validated in brain organoids [227].

The implementation of CRISPRn screens is facilitated by several publicly available CRISPRn libraries with various designs and throughput. Since genome-wide screens in CRISPR models are expensive and laborious, minimized library designs with double sgRNAs have been designed and implemented in hPSCs [228]. While very powerful, CRISPRn screens do not always lead to functional KO (either because of in-frame indels or compensatory mechanisms) and

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have off-targets effects: great care should be taken when using libraries that were not designed using the most up-to-date knowledge about most effective and specific sgRNA designs. Most crucially, strong activation of p53 poses a major challenge to implementing CRISPRn screens in hPSCs [67].

CRISPR interference

CRISPRi screens in hPSC models have been performed both as arrayed (23 genes analyzed by imaging [139]) and pooled (30–240 genes analyzed by Perturb-seq or CROP-seq; ~ 2000 to all coding and non-coding genes analyzed by bulk sgRNA counts; Table 2). Constitutive screens can be performed by transducing a lentiviral sgRNA library in hPSCs expressing a CRISPRi effector, for instance, PB-delivered dCas9-KRAB-MeCP2 [229]. Conditional screens, instead, can be implemented in hPSCs engineered with a Dox-inducible dCas9-KRAB either by lentiviral transduction [230] or *AAVS1* genome editing [138]. These methods were validated during neural and endoderm differentiation, respectively, using proliferation and FACS-based screens, as well as Perturb-seq and CROP-seq.

To overcome the aforementioned limitations in hPSC-derived cell numbers and homogeneity, CRISPRi screens can be performed in forward programmed cell types. This strategy combines *AAVS1*-targeted, Dox-inducible TF overexpression and a constitutively expressed CRISPRi effector genome edited into the *CLYBL* locus: either dCas9-BFP-KRAB, or the TMP-inducible version of the same protein (Fig. 2C; [139]). Double genome-edited hPSCs are transduced with one or more sgRNA, selected, and induced to differentiate by Dox treatment; in the TMP-inducible system, TMP is added at the desired stage of development to induce KD, which is in principle reversible. The method was demonstrated in forward programmed neurons through both arrayed and pooled screens (up to genome wide for bulk sgRNA counts), including for CROP-seq experiments [139,140]. The approach was also applied for pooled and single-cell CRISPRi screens in forward programmed astrocytes [141] and microglia [142].

CRISPRi screens can potentially overcome the main limitations of CRISPRn and RNAi screens, and provide a promising orthogonal strategy. CRISPRi has reduced off-target effects, can reach high levels of KD (90–100%) [137], and it is less toxic than CRISPRn in cells sensitive to DNA damage such as hPSCs [67]. Existing conditional approaches have, however, only been validated in ectodermal lineages and, as discussed above, there could be caveats associated with the activity of the TRE promoter or the expression of dCas9-BFP-KRAB in certain lineages [143].

Gain-of-function screens

Gain-of-function screens in hPSC models have been predominantly used to test the effect of gene overexpression on early lineage specification, particularly due to the difficulties in conditionally regulating GoF. Recent advances in CRISPRa technologies are opening the doors to more nuanced GoF assessments of gene function also in development and disease.

Transgenesis

Gain-of-function screens in mammalian cells historically relied on overexpression of cDNAs (i.e., with ORFeome expression libraries [231,232]). Arrayed transgenesis screens can be performed through PB delivery of Dox-inducible cDNA in hPSCs stably expressing rtTA [233]. The approach was validated through generation of 2135 hPSC clones conditionally expressing a total of 714 transgenes, 511 of which were profiled by RNA-seq.

Pooled transgenesis screens can use lentiviruses expressing barcoded cDNAs for subsequent deconvolution of the data by sc-RNA-seq [234]. In this method, each ORF is paired with a unique 20 base pairs barcode sequence located upstream of the lentiviral 3′-long terminal repeat: this yields a polyadenylated transcript containing the barcode proximal to its 3′ end, which can be detected by sc-RNA-seq methods that count 3’ UTRs. The approach was exemplified by analyzing up to 431 TFs and their effect on hPSC fitness and differentiation in the same experiment [234]. Pooled expression of a larger number of Dox-inducible TFs is facilitated by using PB transposons, which also leads to a higher number of integrations per cell [235]. The approach was exemplified by expressing a TFome of 1732 splice isoforms of 1564 TFs, 290 of which were found to induce differentiation by bulk counts after FACS sorting [235]. The same method can be extended to single cell analyses of transcriptome and TF barcodes from the same cell [165,236].

Open reading frame overexpression outperforms CRISPRa efficiency in hPSC [165], and allows to selectively activate certain splicing isoforms. However, this approach remains challenging to apply at scale due to hurdles in molecular cloning. Moreover, the broad distribution of ORFs sizes poses a challenge for pooled propagation and screening, particularly in lentiviral particles whereby packaging efficiency decreases with increasing vector length [237].

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CRISPR activation

CRISPRa screens in hPSC models have begun to emerge at various scales of throughput (38–100 genes for CROP-seq; 2000 genes to genome wide for bulk sgRNA counts). This can be achieved with a TMP-inducible method analogous to the CRISPRi approach described above, except for being based on a CRISPRa effector (Fig. 6B). Double genome-edited hPSCs are transduced with a sgRNA library, forward programmed into neurons or microglia, and subjected to CRISPRa [140,142].

Compared to transgenesis, CRISPRa pooled screens are easier to deploy as the sgRNA library construction is much simpler. The lower efficiency of CRISPRa that makes it less suitable for forward programming may actually be an advantage for functional genomic studies that could benefit from GoF perturbations in a more physiological range. The integration of results from orthogonal methods such as CRISPRa and CRISPRi can reduce false positives and provide more important insights into gene function [140,142].

Change-of-function screens

Change-of-function screens in hPSC models are challenging due to the low rate of HDR, which limits the efficiency of HR with a library of templates. This difficulty is compounded for loci that are silent in hPSCs as the use of a selective marker operationally linked to the genome edit would require a separate promoter, increasing the cargo size and hence decreasing HDR efficiency. Furthermore, heterochromatin can suppress the activity of constitutive promoters, and decrease the efficiency of HDR. CRISPRa On-Target Editing Retrieval (CRaTER) overcomes these issues by transiently activating the target locus with CRISPRa, so as to allow the selection of cells that undergo HR of a transgene-fluorescent protein fusion using FACS [173]. The approach was demonstrated to enable saturation mutagenesis of five amino acid residues within a mutational hotspot of *MYH7*, a cardiomyopathy-associated gene, which yielded ~ 80% of possible missense variants in hPSCs, 19 of which were clonally genotyped for further characterization in hPSC-CMs.

The use of site-specific recombinases can circumvent the limited rate of HDR in hPSCs. STRAIGHT-IN (serine and tyrosine recombinase-assisted integration of genes for high-throughput investigation) utilizes a three-step procedure to create a landing pad for Bxb1 integrase, recombine a large payload, and remove ancillary sequences with Cre recombinase [238]. The approach is lengthy and requires clonal genotyping, but can replace fragments of at least ~ 50 kb leaving minimal scars, enabling GoF screens in an endogenous-like context (i.e., preserving alternative splicing regulations). This was exemplified in a small screen of 12 variants for the *KCNH2*, an arrhythmia-associated gene, 10 of which could be clonally isolated and characterized by electrophysiology.

Another approach, DICE (dual integrase cassette exchange), allows complete control over the content, orientation, and copy number of gene transfer by using phiC31 and Bxb1 integrases [239]. The method can insert many modifications at the same locus, and was exemplified in a small scale screen of variants of unknown significance in *TNNT2*, a cardiomyopathy-associated gene (Fig. 6C; [240]). Out of 120 target variants, 14 could be isolated through clonal genotyping and functionally characterized in hPSC-CMs.

While these methods have been so far deployed using clonal genotyping prior to phenotyping, improvements in efficacy and/or refinements in the enrichment strategies could unlock the potential of pooled screens with larger throughput.

Design and analytical methods

Robust bioinformatics methods are pivotal both for the design of screening libraries, particularly for RNAi and CRISPR methods, and for extracting biological insight from noisy pooled screening data. Moreover, single cell screens require specialized analytical methods.

RNA interference screens

Extracting robust biological data from pooled RNAi screens remains challenging due to the likelihood of identifying false-positive hits caused by off-target effects, false-negative hits caused by ineffective RNAi, and variance in sequencing depth [241]. To mitigate these challenges, algorithms can optimize the design of RNAi molecules and predict their silencing efficacy [213,242–244]. However, this does not bypass the need to experimentally validate silencing and use multiple RNAi perturbations per target. Bioinformatic tools can then reliably assess the effect of such perturbations by using gene-ranking algorithms to control for off-target effects and then assigning statistical significance to gene ranks [245,246].

Analytical methods for hit selection in RNAi screens are designed to minimize false positives and false negatives (reviewed in [241]). Simpler statistical approaches identify a reliable threshold for RNAi enrichment/depletion, such as the number of standard deviations from the mean/median, or the more rigorous strictly

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standardized mean difference (SSMD). More complex and flexible tools such as rank-product (Bioconductor) and Bayesian models are computationally intensive but can improve hit selection. ZetaSuite is a recent comprehensive pipeline that implements quality controls and statistical methods, as well as hit selection using a support vector machine (SVM) model, which assigns a screen strength score to each gene [247]. The use of machine learning approaches requires a sufficient number of positive and negative controls.

CRISPR/Cas9 screens

Pooled CRISPR screens pose similar design and analytical challenges. Several methods can support the design of sgRNA libraries to optimize the cost efficacy and specificity of the experiment (reviewed in [248]). sgRNA design can rely on simple homology-based methods or more advanced machine learning approaches, which support reduced off-targets and increased efficiency. Biased off-target detection methods for sgRNA, such as the *in silico* predictive models, require experimental data as extensive as possible to increase the algorithm performance. These machine learning algorithms can be divided into two categories: alignment-based, which predict off-target effects based on sequence homology [249], and scoring-based, which consist of more complex algorithms that assign a score and ranking to possible sgRNAs [250,251]. These methods were based mostly on cancer cell line datasets, but have now been applied to a wide variety of cell types.

The hit identification in dropout/enrichment pooled CRISPR screens is not trivial, as the sgRNA distributions are variable and over-dispersed in both positive and negative controls [252]. Methods that address this issue were developed starting from computational approaches originally developed for pooled RNAi screens [252,253]. CRISPRi and CRISPRa screens in hPSC models can be analyzed by MAGeCK-iNC, which computes *P*-values for sgRNA counts across two populations by also considering the distribution of non-targeting control sgRNAs [139–142].

Single cell screens

Pooled GoF and LoF screens using methods such as Perturb-seq and CROP-seq require *ad hoc* analytical methods (reviewed in [254]). These analyses must determine hits on a complex multidimensional space involving several thousands of genes rather than a single phenotype. Moreover, these expensive screens usually have one or few replicates, are characterized by noisy, zero-inflated, sparse data, and are subject to confusing factors such as off-target and/or inefficient perturbations.

A common and conceptually simple approach to identify the effects of CRISPR perturbations in single cell screens is to deconvolute the multidimensional data into a simpler two-dimensional space, define clusters of cells, and determine whether certain sgRNAs are enriched in one or more of these clusters by hypergeometric or chi-squared test. Alternatively, differential gene expression analyses can be performed between cells with a given perturbation (i.e., gene KD/KO or individual sgRNA) versus control cells; this can be performed for all perturbations and followed up by clustering of differentially expressed genes versus perturbations to identify regulatory modules that can be further studied (i.e., by gene ontology analyses).

Analyses of more nuanced phenotypes can be performed using tools based, for instance, on linear models, topic models, or ranks. MIMOSCA was developed for PERTURB-seq screenings [204], and can be used to identify genes involved in hPSC differentiation [138]. MIMOSCA interprets the combined non-linear effects of multiple factors perturbations on gene expression: it predicts the expression level of each gene as a linear combination of sgRNA effects, fitting the regulatory effect of each guide on each gene and creating a coefficient matrix; then, with a permutation-based test, it evaluates the significance of each coefficient. MUSIC uses a natural language processing method, topic modeling, to derive the biological function “topics” of each cell “document” through gene expression “words”; then, it quantitatively estimates and prioritizes perturbation effects on cell phenotypes at various levels: overall effect topic-specific effect and relationship between perturbations [255]. scMAGeCK is a single cell extension of MAGeCK; it has two analyses mode: scMAGeCK-Robust Rank Aggregation (RRA), which finds perturbations associated to a single marker, and scMAGeCK-LR, a linear-regression-based approach that determines the effect of perturbations considering all genes and is suitable for cells with multiple perturbations [256].

Future directions

The first quarter century in the history of hPSCs revolutionized our ability to study human gene function in development and disease, and set the foundation for potentially transformative clinical applications. While it is impossible to forecast the next 25 years, certain advances are visible on the horizon. Indeed, many technologies optimized in easier-to-work-with cells are likely to make the leap to hPSC models soon. Without the pretense to be comprehensive, we wish to list some

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potential directions that could improve the various aspects discussed in this review.

Improving hPSC editing

While plasmids, viruses, and transposons will continue to be essential tools to deliver nucleic acids in hPSCs and their derivatives—particularly for high-throughput screens—gene editing of GSHs is now firmly the method of choice for precisely and reproducibly engineering hPSCs. Commonly used GSHs are, in large part, a historical legacy, and do not fulfill all of the desired characteristics (i.e., adequate for reliable and safe therapeutic transgene addition; [257]). Moreover, as already discussed, the most popular GSH, the *AAVS1* locus, does not support homogeneous transgene expression with many promoters and can be methylated. In this light, the recent identification of several novel human GSH sites may pave the way to more predictable hPSC genome editing [258–260]. Such GSH candidates were identified through a combination of methods and were rigorously assessed for widely accepted criteria for *bona fide* GSH status, but their use in hPSCs and their derivatives remains to be demonstrated. A larger pool of robust GSHs would also enable the generation of hPSCs with multiplexed edits to engineer sophisticated regulatory networks.

Refining gene manipulation methods

Loss of function

An important addition to the LoF toolbox would be methods to control gene expression post-transcriptionally and at an isoform-specific level with increased specificity and efficacy than existing RNAi methods based on siRNA and shRNAs. For instance, optimized amiRNAs such as the miR-E [261] and miR-3G [109] lead to potent KD even when expressed as single copy, and, being more precisely cleaved, have lower off-target effects compared to shRNAs. They can be flexibly integrated within Pol II-driven mRNAs —easing lineage-specific regulation—or expressed under the control of Pol III promoters—decreasing cassette size [109]. Ultra-potent synthetic amiRNA have been recently developed, which allow KD experiments with virtually no off-targets, outperforming even CRISPRi [262]. Application of amiRNAs to hPSCs have so far been limited to first-generation miR-30 designs [51,98–100], which are less powerful [261].

Another exciting possibility for improved post-transcriptional RNA silencing in hPSCs is presented by methods based on type-VI CRISPR/Cas systems (reviewed in [263]). For instance, Cas13d can be directed by CRISPR RNA to hydrolyze specific RNAs, with increased efficiency and specificity than RNAi [264]. This strategy has been recently deployed in hPSC-derived neurons [265], but offers strong potential for many other cell types.

With regard to transcriptional silencing methods, CRISPRoff has not yet found widespread use in hPSC models, but promise to facilitate LoF studies as well as, possibly, clinical applications requiring stable gene silencing and preferring a non-genotoxic approach that only modifies the epigenome. Since CRISPRoff does not require sustained expression of the repressor to achieve long-term silencing, it has lower risk long-term toxicity compared to conventional reversible CRISPRi, which requires chronic expression of its silencing effector.

Gain of function

A long standing quest is developing conditional GoF methods that are active not only in hPSCs but also in all of their derivatives. Indeed, as already discussed, the widely used Dox-inducible TRE promoter is often poorly active after hPSC differentiation. Besides the use of insulators, which has already been explored with some degree of success, TRE promoter stability in hPSC models could be improved by adding an universal chromatin opening element (UCOE), a strategy that was demonstrated to maintain Dox inducibility in other models [266]. Stably integrated Dox-inducible transgenes have been mostly inserted into the *AAVS1* locus, but as discussed in the previous section, other GSHs may prove more suitable. Alternatively, other -Dox-responsive promoters that do not rely on a CMV minimal promoter may prove more active in certain hPSC-derived cell types [267].

Improvements in the performance of CRISPRa methodologies may come in many ways, first and foremost the development of novel effectors, such as CRISPRon. Strong potential lies within the growing number of type-II Cas9 and type-V Cas12 proteins [268], some of which may be coaxed into even more effective CRISPRa methods than SpCas9. Methods that recruit activator domains using RNA aptamers within the sgRNA, such as CRISPR-SAM, could prove advantageous to efficiently deliver CRISPRa effectors that are stably expressed in hPSCs and their derivatives.

Change of function

CRISPR BE and PE have taken center stage in CoF experiments in hPSC models. Improvements in the

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specificity and activity of these methods will broaden their applicability, particularly in the context of clinical applications. The development of conditional BE/PE methods would enable the study of mutations that are incompatible with the pluripotent state and/or at specific stages of development. Cas13-based RNA BE has also emerged as a promising method that could enable for the first time post-transcriptional GoF experiments (reviewed in [263]).

Combinatorial methods

The development of robust methods that combine LoF, GoF, and/or CoF to engineer sophisticated regulatory networks in hPSC models would enable refined mechanistic studies of gene function, and could allow the engineering of complex cell functions. This can be achieved using either orthogonal CRISPR/Cas9 systems, for instance, to support combinatorial CRISPRa and CRISPRi in the same cell using different sgRNAs specific for two catalytically inactive Cas9 proteins fused to an activator or repressor, respectively [269]. Alternatively, the use of RNA aptamers to recruit effectors can enable more nimble combinatorial approaches requiring a single dCas9 protein, leaving the sgRNA scaffolds to dictate whether a repressor or an activator should be recruited at a given locus [270,271].

Extending functional genomic screens

Besides advancements in the underlying methods such as those discussed above, functional genomic screens would benefit from more robust underlying cell models. For instance, the use of hPSC forward programming to generate large numbers of highly pure and homogeneous differentiated cells would facilitate genome-wide screens, as already demonstrated in neurons, astrocytes, and microglia [139–142]. Another aspect where increased homogeneity would reduce experimental noise is the delivery of perturbations: screens based on isogenic genome editing through HR or site-specific recombination could improve specificity and sensitivity, and lower costs by reducing the number of cells needed to obtain statistically significant results. Methods that account for hPSC clonal heterogeneity would be advantageous, as outlier populations resulting from genetic and/or epigenetic aberrations could be easily filtered out.

Several functional genomic methods are ripe for deployment in hPSC models; for instance, CaRPool-seq—which leverages on Cas13d to perform single cell post-transcriptional LoF screens [272]; CRISPR BE and PE screens—which enable pooled CoF mutagenesis screens [273–275]; Perturb-ATACseq [276], Spear-ATAC [277], and CRISPR-sciATAC [278]—which combine CRISPR pooled screens with single-cell assay for transposable accessible chromatin by NGS (sc-ATAC-seq) for high-throughput assessment of epigenetic regulations. Besides such existing approaches, novel CRISPR screens based on single-cell multimodal omics methods (reviewed in [279]), could pave the way to an unprecedented multilevel assessment of gene function. All of this, of course, will require parallel improvements in bioinformatics methods to design screens to contain costs, and, most importantly, extract maximal biological insight.

Conclusions

The recent rise of transformative technologies such as CRISPR/Cas and single cell genomics has taken the young field of hPSC biology by storm, blurring the boundaries between fields and accelerating the pace of discoveries to breakneck speed. This rollercoaster is unlikely to slow anytime soon, and those able to keep up with its journey will have a unique opportunity to decode human development and disease and encode the next generation of cell-based medicines.

Acknowledgements

We thank Johannes Zuber for advice on RNAi technologies, Daniel Yang and Clayton Friedman for advice on CoF screens, William G. Bernard for advice on gene editing, and former colleagues in Ludovic Vallier and Chuck Murry's groups for insightful discussions on the subject matter. All figures were created using BioRender.com. This work was supported by the Giovanni Armenise-Harvard Foundation Career Development Award 2021, the Additional Ventures Single Ventricle Research Fund 2021, and the FEBS Excellence Award 2022 (grants to AB), and by PON R&I Azione IV.4 & IV.5 – FSE REACT-EU (Ph.D. fellowship to FS and SBo).

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