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Review

# Advances in human pluripotent stem cell reporter systems

Lesly Puspita,[1,3] Virginia Blessy Juwono,[1,2,3] and Jae-won Shim[1,2,*]

## SUMMARY

**The capability of human pluripotent stem cells (hPSCs) to self-renew and differentiate into any cell type has greatly contributed to the advancement of biomedicine. Reporter lines derived from hPSCs have played a crucial role in elucidating the mechanisms underlying human development and diseases by acting as an alternative reporter system that cannot be used in living humans. To bring hPSCs closer to clinical application in transplantation, scientists have generated reporter lines for isolating the desired cell populations, as well as improving graft quality and treatment outcomes. This review presents an overview of the applications of hPSC reporter lines and the important variables in designing a reporter system, including options for gene delivery and editing tools, design of reporter constructs, and selection of reporter genes. It also provides insights into the prospects of hPSC reporter lines and the challenges that must be overcome to maximize the potential of hPSC reporter lines.**

## INTRODUCTION

Human pluripotent stem cells (hPSCs) have emerged as a powerful and versatile tool in biological research and regenerative medicine. Human embryonic stem cells (hESCs) are derived from the inner cell mass of the human embryo, whereas human-induced pluripotent stem cells (hiPSCs) are somatic cells reprogrammed to pluripotency. Based on the capability of hPSCs to self-renew and differentiate into the three germ layers, various protocols have been established to differentiate them into neuronal, lung, intestinal, kidney, and pancreatic cells.[1–5] In Parkinson disease, the potential of hESC-derived midbrain dopamine (mDA) neurons as a source for transplantation has been continuously explored in clinical trials.[6] In addition to neurodegenerative diseases, hPSC derivatives have been used in clinical trials to treat cardiovascular diseases, diabetes, and certain types of cancer and tumors.[7] Patient-derived hPSCs provide a relevant human-based platform that can improve the success rate of drug development and have greatly contributed to our understanding of various disease mechanisms.[8,9]

Reporter systems are genetically modified cell lines that express reporter genes that can be visualized and quantified. Reporter genes are expressed under the control of promoters that respond to specific biological signals and environmental cues.[10] Reporter cell lines provide a non-invasive tool for observing cellular dynamics without the need for sample destruction. Therefore, different assays can be performed simultaneously with the same population, thereby reducing the heterogeneity factor between samples. Many techniques for establishing reporter systems were first developed in animal models. Reporter lines have important implications in various research fields owing to their ability to track and monitor cell behavior in real time. Although the application of reporter systems in living humans is difficult, hPSC technology enables the generation of reporter systems in human-based platforms.

This review explores the development of reporter cell lines from hPSCs and their applications in various research fields, highlighting their versatility and crucial roles in advancing our understanding of human development and disease mechanisms.

## APPLICATIONS OF hPSC REPORTER LINES IN VARIOUS RESEARCH FIELDS

In the evolving landscape of biomedical research, hPSC reporter lines have emerged as indispensable tools for revealing the mechanisms underlying human development and disease onset. Reporter genes are integrated into specific loci within hPSCs to illuminate the dynamics of gene expression and cellular behavior in unprecedented detail. Consequently, hPSC reporter lines are used in a broad spectrum of research domains (Figure 1). In this review, we summarize the various applications of hPSC reporter lines.

### Cell purification for transplantation and other downstream purposes

Despite the promising results of fetal-derived grafts in regenerative medicine, the rarity, heterogeneity, and ethical concerns associated with the utilization of human fetal cells limit their widespread clinical applications.[11] hPSCs offer a promising alternative source of transplantable cells. However, undesired populations must be sorted to ensure the safe and successful integration of hPSCs into donors.[12] Heterogeneity in

[1]Soonchunhyang Institute of Medi-bio Science (SIMS), Soonchunhyang University, Cheonan-si 31151, Korea
[2]Department of Integrated Biomedical Science, Soonchunhyang University, Cheonan-si 31151, Korea
[3]These authors contributed equally
*Correspondence: shimj@sch.ac.kr
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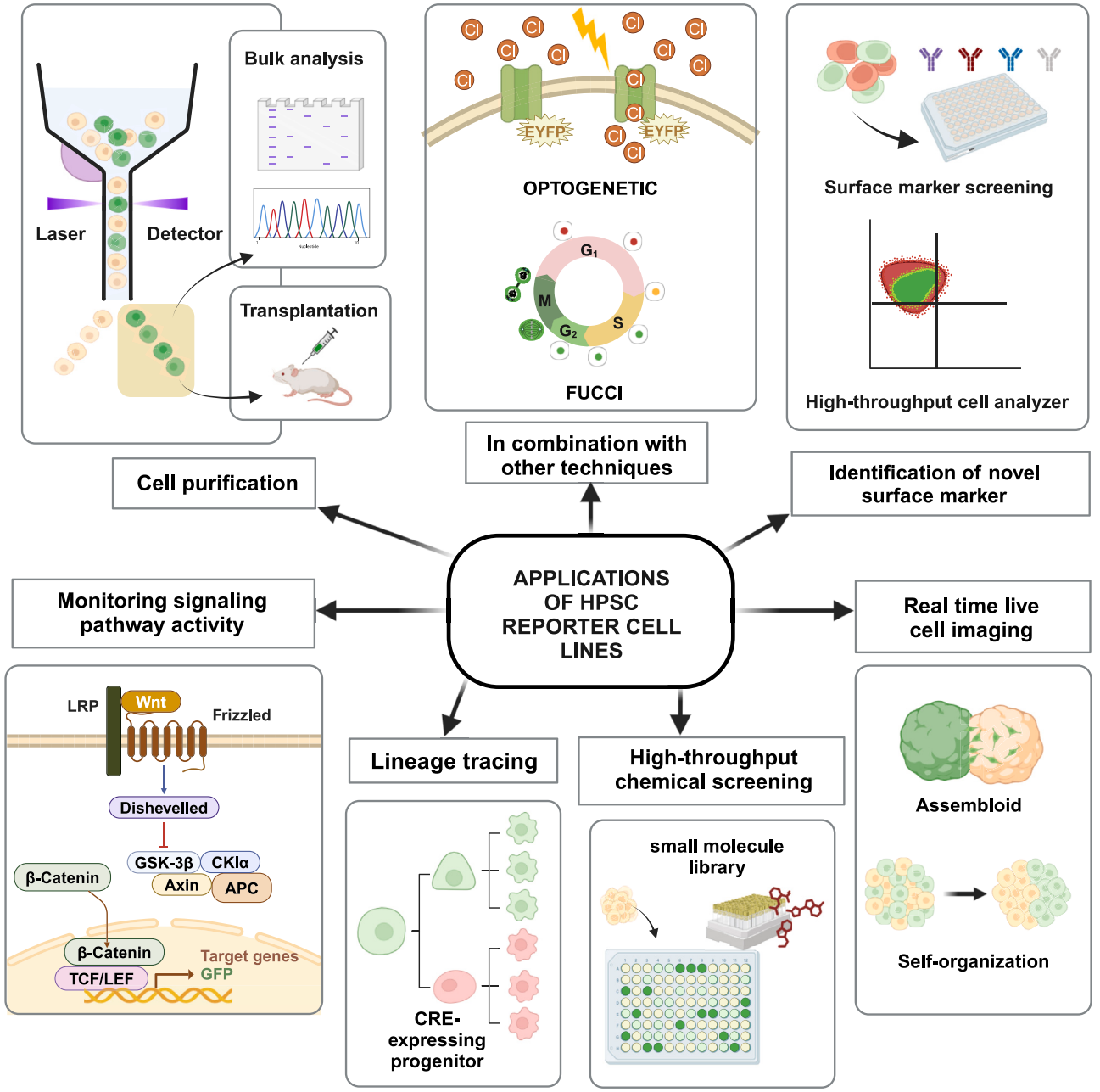


**Figure 1. Diverse applications of hPSC reporter cell lines in biomedical research**

hPSC-derived reporter cell lines have been utilized in various biomedical applications, such as cell purification, tracking cell lineages, novel cell surface marker identification, real-time live cell imaging, high-throughput screening, and monitoring of signaling pathway activity. These cell lines have also been instrumental in complementing other techniques, such as FUCCI and optogenetics.

cell populations after differentiation from hPSCs also limits interpreting data from bulk analysis methods, such as western blot, RNA sequencing, and real-time quantitative PCR. Isolating the desired populations is often necessary to improve data reliability and accuracy, particularly when the expected change is small and potentially masked in a heterogeneous population. Fluorescence-activated cell sorting (FACS) allows the isolation of the desired cell populations based on their fluorescence properties. Fluorescence-conjugated antibodies that recognize cell surface proteins specific to the target population can be used for such sorting purposes.[13] However, not every cell type has a distinct cell surface protein that can be recognized using commercially available antibodies. Another strategy for labeling a specific group of cells involves inserting a fluorescent reporter into a cell-type-specific gene.[14–16]

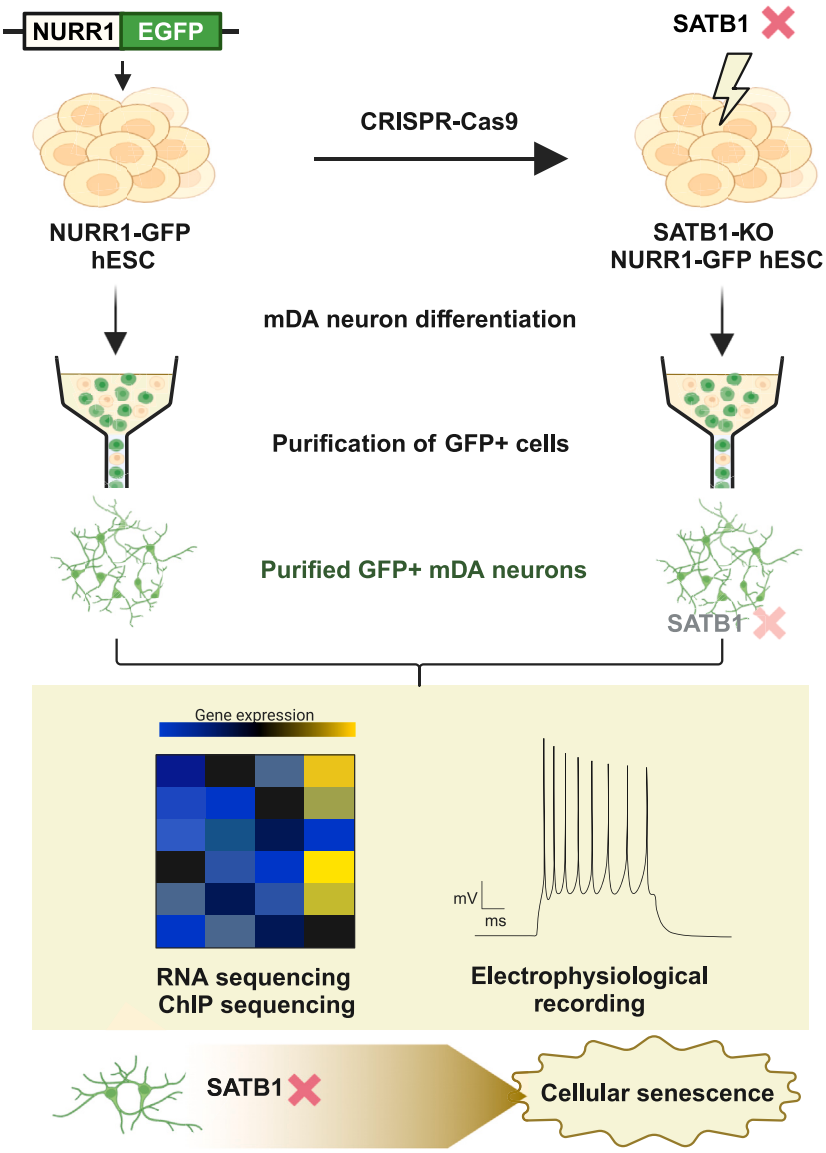


**Figure 2. Utilization of the hPSC reporter line in Parkinson disease modeling**

A reporter line expressing GFP in conjunction with NURR1 was subjected to a secondary genetic modification to knock out SATB1, a gene associated with Parkinson disease risk. Subsequent analysis following the purification of GFP-expressing mDA neurons revealed the crucial role of SATB1 in preventing cellular senescence in these neurons.

This purification step is often important for the differentiation of specialized cell types, such as mDA neurons, from hPSCs. Using green fluorescent protein (GFP) expressed under endogenous nuclear-receptor-related factor 1 (NURR1) promoter, a postmitotic mDA neuron marker, enabled the isolation of hPSC-derived mDA neurons with 98% purity for applications in bulk transcriptome analysis and Parkinson disease modeling study (Figure 2).[17] Two other reporter lines were used to sort hPSC-derived populations at different stages of mDA neuronal development. In addition to the functionality of mDA neurons, determining the ideal developmental stage of the graft is crucial for successful transplantation. Using reporter lines LIM homeobox transcription factor 1 alpha (LMX1A)-GFP and paired-like homeodomain 3 (PITX3)-GFP, a previous study revealed that the ventral midbrain progenitor stage (LMX1A+) allows faster recovery and appropriate innervation upon transplantation than the mDA neuron precursor stage (PITX3+).[15]

The need for a sorting step can also be seen in the generation of serotonin neurons from hPSCs. Existing protocols vary in their yield, with one protocol showing approximately 60% and another showing 30%–40% efficiency.[4,18] Using enhanced green fluorescence protein (EGFP)

expressed under the tryptophan hydroxylase 2 promoter can increase the percentage of hPSC-derived serotonin neurons from approximately 22.08%–95.43%.[14]

## Identification of novel cell surface markers

The presence of foreign DNA sequences in the donor cells raises concerns in clinical transplantation studies. To facilitate transgene-free grafts and enhance clinical applicability, identifying an endogenous surface protein for isolating desired cells is critical. Using reporter cell lines for the initial enrichment step can accelerate this process, as shown by the utilization of an LMX1A-GFP reporter line to identify a novel surface marker for hPSC-derived midbrain progenitors.[19] Differentiated populations were first sorted based on the GFP signal to isolate midbrain progenitors. Subsequent transcriptome analysis led to the discovery of a novel surface marker, trophoblast glycoprotein. This marker can enrich ventral midbrain dopamine precursors for Parkinson disease transplantation.[19] Additionally, reporter cell lines have been utilized alongside a commercially available library of human cell surface markers.[20] For instance, the myogenic factor 5-GFP reporter line facilitated the discovery of novel surface markers for skeletal myogenic progenitors. Upon staining with 242 monoclonal antibodies that recognize human cell surface proteins, the cells were subjected to a high-throughput screening cell analyzer. Two markers, cluster of differentiation (CD) 10 and CD24, demonstrated consistent positive and negative correlation with GFP expression, respectively. These markers were thus proposed as candidate surface markers to isolate skeletal myogenic progenitors, a development that could accelerate clinical applications for treating severe muscle disorders such as sarcopenia and muscular dystrophy.[20] A recent notable study utilized the NURR1-GFP reporter line with a panel of 385 validated antibodies to identify novel surface markers of postmitotic DA neurons, crucial for Parkinson disease clinical trials.[21] A high-throughput flow-based analysis identified CD49 and CD184 as effective negative and positive selection markers, respectively, resulting in the highest ratio of postmitotic mDA neurons.[21] Altogether, the hPSC reporter system is an essential tool in the discovery of novel surface markers, pivotal for the future clinical application of hPSC-derived cells (Figure 3).

## Lineage tracing

The complexity of multicellular organisms depends on the capability of embryonic cells to multiply and differentiate into various cell types. Understanding the relationship between mature cells and their progenitors is key to establishing hPSC differentiation protocols. Gene engineering enables spatiotemporal control over the expression of a reporter gene, allowing progenitor cells to be marked and monitored in real time and the lineage of their descendant cells to be traced. In a previous study, a dual reporter system was generated to confirm the origin of different types of neural crest that would later develop into the enteric nervous system.[22] Fluorescent genes *GFP* and *tdTomato* were inserted into the loci of neuro-mesodermal progenitor (NMP) markers *brachyury* (*T*) and *SRY-box transcription factor 2*, respectively. Three days after the initiation of differentiation, double-positive NMP-like cells were enriched using FACS and then further differentiated. Robust generation of sacral neural crests (SNCs) from the sorted populations confirmed that SNCs originate from NMP-like precursors.[22]

In another study, Cre-recombinase was used with a dual reporter system to understand nephrogenesis in hPSC-derived kidney organoids. To confirm the previous results in animal models, the system was designed to trace the lineage of sine oculis homeobox homolog 2 (SIX2)+ nephron progenitors. The Cre recombinase gene was inserted into the endogenous locus of SIX2, whereas a dual fluorescent reporter cassette consisting of a loxP-flanked *EGFP* next to the *mCherry* gene was inserted into the locus of the housekeeping gene *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*). *EGFP* was stably expressed in the reporter cells in the absence of Cre (SIX2CRE/CRE: GAPDH dual iPSCs). As the organoids formed, a subset of progenitor cells began to express SIX2 and Cre recombinase, irreversibly switching the EGFP signal into mCherry. The expression of mCherry in the descendant cells mapped the contribution of SIX2+ progenitors to nephron formation.[23]

## Real-time live cell imaging

Analysis of cellular development and disease phenotypes is not limited to the downregulation and upregulation of protein expression. It also involves migration, neurite extension, protein localization, membrane depolarization, and other cellular dynamics. Reporter systems enable the monitoring of cellular phenomena in living cells. Progenitor cells migrate as they mature and differentiate. Among them are retinal progenitor cells, which are precursors of photoreceptor cone cells that form the outer nuclear layer of the human retina.[24] Cone cells are responsible for color vision, and impairment of their formation results in several types of vision abnormalities, including color blindness. In a previous study, the *mCherry* gene was inserted into the locus of the endogenous *guanine nucleotide-binding protein subunit gamma-T2* gene to observe cone cell development in real time.[25] Results revealed a red fluorescent signal that was expressed exclusively by post-mitotic cone precursor cells. Upon differentiation into three-dimensional retinal organoids, the migration of cone precursor cells was captured along the radial axis of retinal organoids. This observation challenges the previous hypothesis that cone precursor cells do not undergo any migration, based on the shared location of their formation and final mature sites.[25]

Reporter systems have also contributed to the establishment of assembloids, a fusion of two or more spheroids that are often patterned into different regional identities.[26,27] Labeling one or both spheroids with fluorescent molecules allows tracking the cell origin in the assembloids. The first publication about the generation of assembloids successfully demonstrated in an *in vitro* system that interneurons migrate from ventral-forebrain-patterned spheroids into dorsal-forebrain-patterned spheroids. In this study, the reporter gene *EGFP* was inserted into the locus of *Dlxi1/2b* to specifically label the medial ganglionic eminence (ventral forebrain origin) and the GABAergic interneurons of its descendants.[26] Defects in the migration of cortical interneurons are involved in Timothy syndrome, a severe neurodevelopmental disorder. The same reporter line was used to study the pathogenic mechanisms of Timothy syndrome.[27]

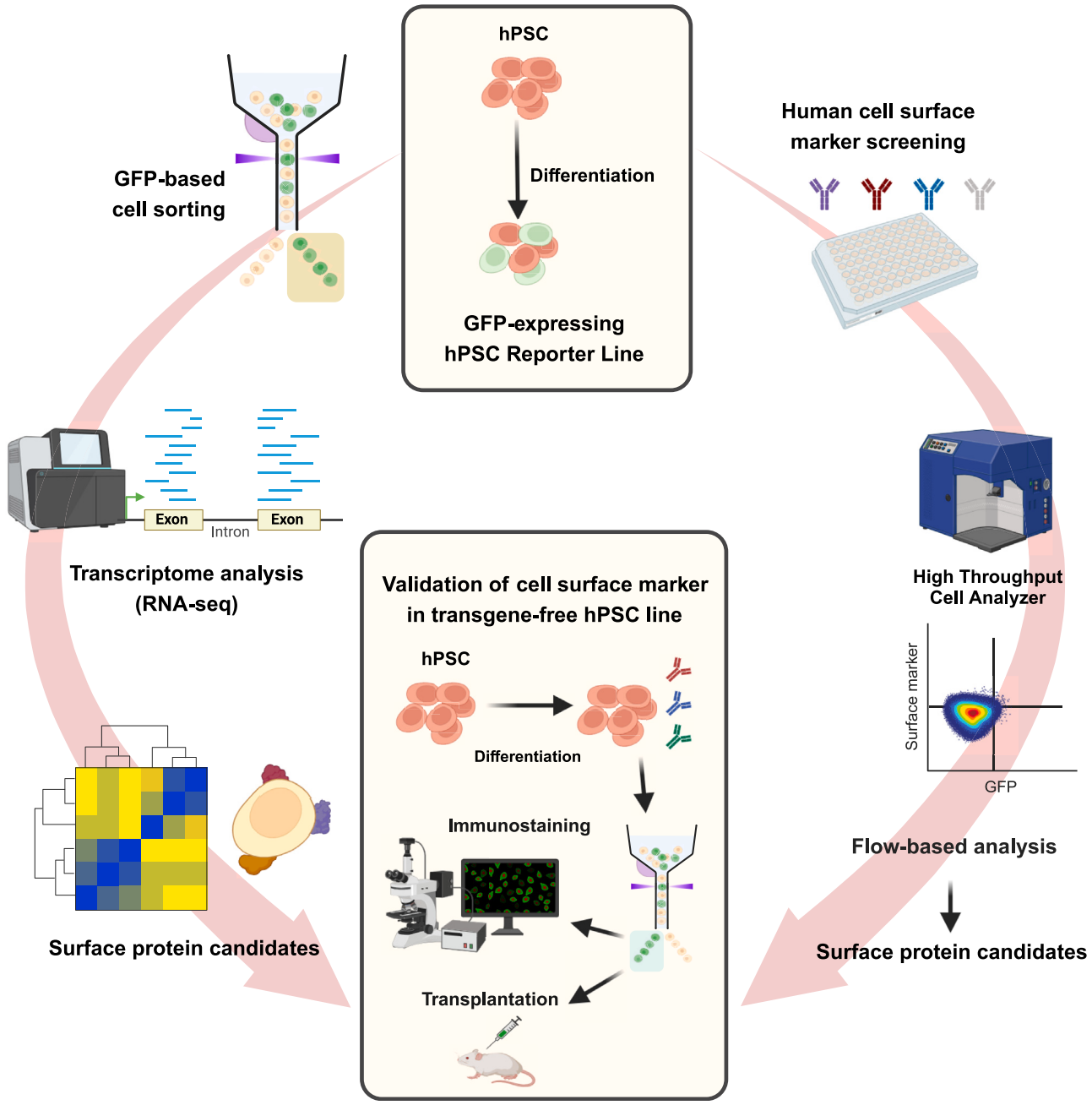


**Figure 3. Identification of novel surface marker with hPSC reporter line**

Reporter genes, driven by specific promoters, aid in the initial purification step prior to RNA sequencing for identifying distinct surface proteins (left arrow). Alternatively, a panel for human cell surface marker screening can be used for this purpose. The reporter system enables the identification of surface markers that are uniquely expressed on the reporter-expressing cells within a heterogeneous population (right arrow).

## High-throughput chemical and drug screening

High-throughput screening allows the simultaneous testing of a large number of chemicals to determine their effects on cellular activities. Between 2018 and 2021, only approximately 10% of drugs from clinical trials were approved by the Food and Drug Administration (FDA), indicating the inefficiency of animal models in predicting the efficacy and safety of drug candidates.[28] By contrast, hPSCs provide versatile human-based models that can be reproduced and designed to match the requirements of an assay. Reporter line technology allows multiple analyses to be performed simultaneously on the same population. Li et al. developed a triple-reporter system for the massive screening of two libraries of bioactive compounds for their myelination-promoting capacity using hPSC-derived oligodendrocyte precursor cells (OPCs) as

subjects.[29] Oligodendrocytes are glial cells in the central nervous system that play a central role in axon myelination. Previous studies explored the transplantation of OPCs for the treatment of spinal cord injury and multiple sclerosis.[30,31] The *tdTomato* and *Thy1.2* genes were inserted into the locus of *platelet-derived growth factor alpha receptor* by using clustered regularly interspaced short palindromic repeats (CRISPR) to allow the visualization and isolation of OPCs. Maturation of OPCs into functional oligodendrocytes was observed based on the expression of *superfolder GFP* inserted into the locus of *proteolipid protein 1*. Lastly, to measure myelination capacity, the luciferase gene was inserted into the locus of *myelin basic protein*, which is expressed at the late stage of oligodendrocyte differentiation. This triple-reporter system has been used to simultaneously test the myelination-promoting activity of approximately 2,500 compounds at various doses and time points. The study listed the top 15 hit compounds from the two libraries, among which two had never been previously linked to the induction of oligodendrocyte maturation or myelination.[29]

### Visualization of signaling pathway activity

Signaling pathways are a battery of molecular events that occur in cells in response to cues or stimuli. Extracellular cues affect cells via outer membrane receptors that relay signals to modulate the cellular machinery that executes the appropriate response.[32] The targets of signaling pathways often include the regulation of transcription factors; hence, the activation or inhibition of a specific signaling pathway can be observed by monitoring the expression of a downstream target gene. Several signaling pathway reporter lines, including Wnt, BMP, and transforming growth factor β (TGF-β) signaling, have been established in murine cells.[33–35] When this article was written, only the Wnt signaling reporter had been published for hESC and hiPSC lines.[36] The cells were transduced with a lentivirus to express GFP under the regulation of consensus T cell factor/lymphoid enhancer factor (TCF/LEF) binding sequence to monitor the activation of the canonical Wnt/β-catenin pathway. The pivotal role of Wnt signaling during the differentiation of cardiomyocytes from hPSCs was confirmed using these cell lines, which led to the establishment of an improved and scalable protocol for generating cardiomyocytes for wider clinical applications.[37,38]

### Other notable applications of hPSC reporter lines

The advancement of numerous recent and sophisticated innovations in cellular biology has been closely associated with the utilization of hPSC reporter systems. Among these, optogenetic and fluorescence-based cell-cycle indicators (FUCCIs) have significantly contributed to numerous discoveries in various research fields.

### Optogenetics

Optogenetics is a technique used to manipulate cellular activity by genetically modifying cells to express proteins that change their conformation in response to light. Optogenetic proteins are developed from photoreceptor proteins that are naturally expressed in certain species of algae and bacteria.[39,40] Given its capability to alter the membrane potential rapidly and reversibly, optogenetics allows the precise modulation of neuronal depolarization, cardiac cell excitability, and insulin secretion.[41–43] Because the optogenetic protein itself does not release observable light, the insertion of optogenetic genes into hPSCs is usually coupled with a fluorescent gene under the same promoter to mark its expression. In a previous study, the inhibitory chloride pump halorhodopsin and enhanced yellow fluorescent protein (EYFP) were combined under the regulation of the pan-neural marker synapsin to control neural activity and dopamine release from hESC-derived dopaminergic neurons post-transplantation in mice.[44] The expression of EYFP helped track the halorhodopsin-expressing graft in the transplanted animal model, allowing the light-induced inhibition of dopamine release, which reversed the rescuing effect of the graft and reintroduced motor dysfunction.[44]

In 2013, Bugaj et al. demonstrated that the application of optogenetics is not limited to the modulation of membrane potential.[45] They activated Wnt signaling through light stimulation by fusing the photoreceptor cryptochrome 2 from *Arabidopsis thaliana* with the cytosolic unit of the Wnt signaling coreceptor low-density lipoprotein receptor-related protein 6 (optoWnt).[45] This system was then used to study the contribution of Wnt signaling activation to the self-organization of hPSCs. mCherry-expressing optoWnt and wild-type hPSCs were mixed and cocultured. After blue light stimulation, mCherry-positive optoWnt cells and their wild-type counterparts migrated in opposite directions, forming clear boundaries between the two populations.[46]

### FUCCI

FUCCI is a genetic tool used in cellular imaging to monitor changes in cell-cycle dynamics.[47] FUCCI typically consists of the fluorescent proteins monomeric Kusabira-Orange 2 (mKO2) and monomeric Azami Green (mAG) fused with the ubiquitination domains of human Cdt1 (mKO2-hCdt1) and geminin (mAG-hGem), respectively. The presence of Cdt1 proteins marks the G1 phase, in which they play a crucial role in licensing the chromosome to initiate DNA synthesis. However, upon DNA replication, Cdt1 is ubiquitinated by SCFSkp2 E3 ubiquitin ligase and degraded by the proteasome during the S and G2 phases. Meanwhile, geminin plays an important role during the S phase by inhibiting the assembly of the pre-replication license complex. During the G1 and M phases, geminin is ubiquitinated by APCCdh1 E3 ubiquitin ligase and degraded during the G1 and M phases. The dynamics of these two proteins result in the reporter lines exhibiting green and red fluorescence signals during the S/G2/M and G1 phases, respectively.[47] This system has been applied to hPSCs to study the influence of cell division on endodermal formation, epigenetic transition, and cell-cycle-related molecular mechanisms that occur in the early stages of endodermal fate acquisition. FUCCI genes were inserted into the hESC genome using the pTP6 system to generate stable and randomly integrated clones. The fluorescent signals enabled the sorting of cells at different cell-cycle stages for subsequent analyses, such as RNA-seq, CHIP-seq, and ATAC-seq.[48,49]

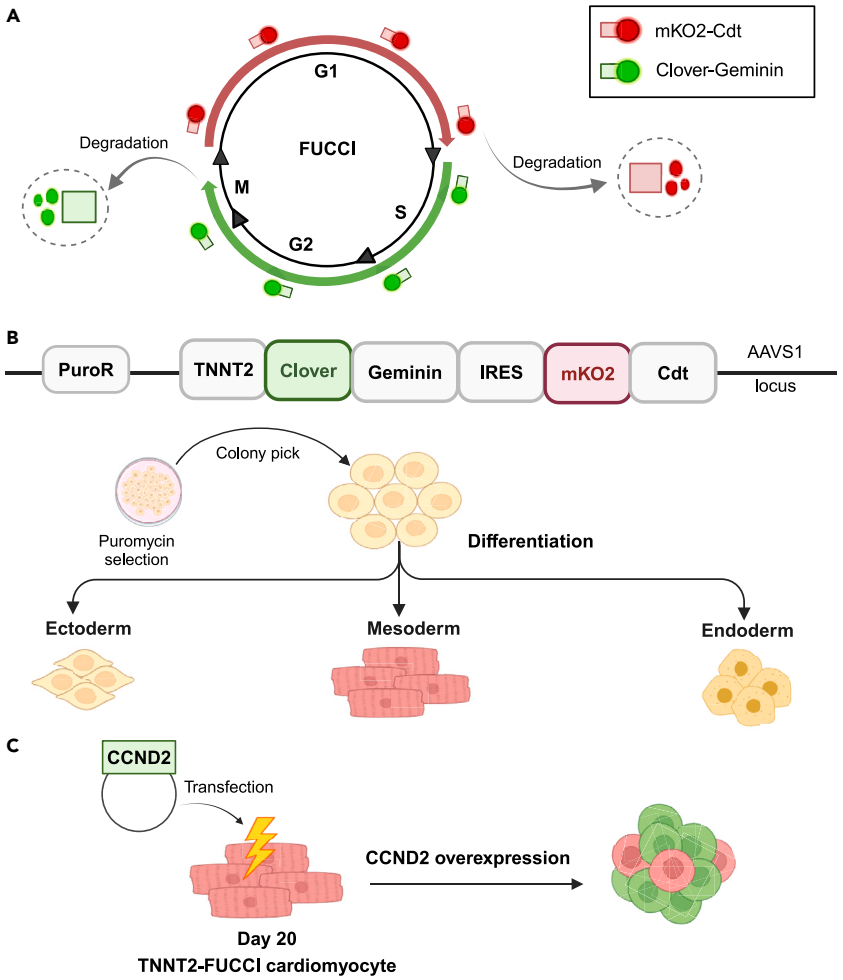


**Figure 4. Monitoring cell-cycle dynamics during hPSC differentiation with FUCCI**

(A) The mKO2-Cdt1 (red) fusion proteins are expressed during the G1 phase and subsequently degraded during the S/G2 phase, whereas Clover-Geminin (green) proteins are expressed during the S/G2 phase and degraded during the G1 phase.

(B) A cassette containing the TNNT2-FUCCI construct was inserted into the AAVS1 safe harbor site. Following colony selection and genotyping validation, TNNT2-FUCCI hPSCs were differentiated into three lineages: mesoderm (cardiomyocytes), endoderm, and ectoderm. Only cardiomyocytes exhibited expression of mKO2-Cdt (G1 phase), indicating that most of these cells were not proliferative.

(C) Overexpression of CCND2 resulted in an increased green Clover signal (S/G2 phase), indicating enhanced cell proliferation.

A previous study developed lineage-specific FUCCI reporter lines from hPSCs, in which the Clover-Geminin and mKO2-hCdt1 proteins were regulated by the cardiac-specific promoter troponin T2, cardiac type (TNNT2). The all-in-one FUCCI construct TNNT2-Clover-Geminin-IRES-mKO2-Cdt1 was inserted with CRISPR/Cas9 into the safe harbor locus adeno-associated virus integration site 1 (AAVS1) to provide a stable transgene that expresses FUCCI proteins upon differentiation into cardiomyocytes (Figures 4A and 4B). The establishment of this reporter system led to the discovery of a novel regulator that stimulates cell-cycle reentry for cardiac tissue regeneration (Figure 4C).[50]

## DESIGNING hPSC REPORTER LINES

Advances in genetic and molecular engineering as the foundation of reporter systems, aided by the continuous exploration for novel and improved reporter designs, have made major contributions to the progression of research with hPSCs. Depending on the purpose of the study and the available resources, hPSC reporter systems can be designed in various manners to match the needs of the study (Figures 5 and 6). In this study, we examined several approaches to designing hPSC reporter lines.

### Insertion of multiple copies of reporter cassette with viral gene delivery

Viral transduction is a popular choice for genetically modifying hPSCs owing to its simplicity and effectiveness in producing high transgene expression.[51] Delivery with retroviruses and lentiviruses allows the stable and random insertion of reporter genes into genomes. With this

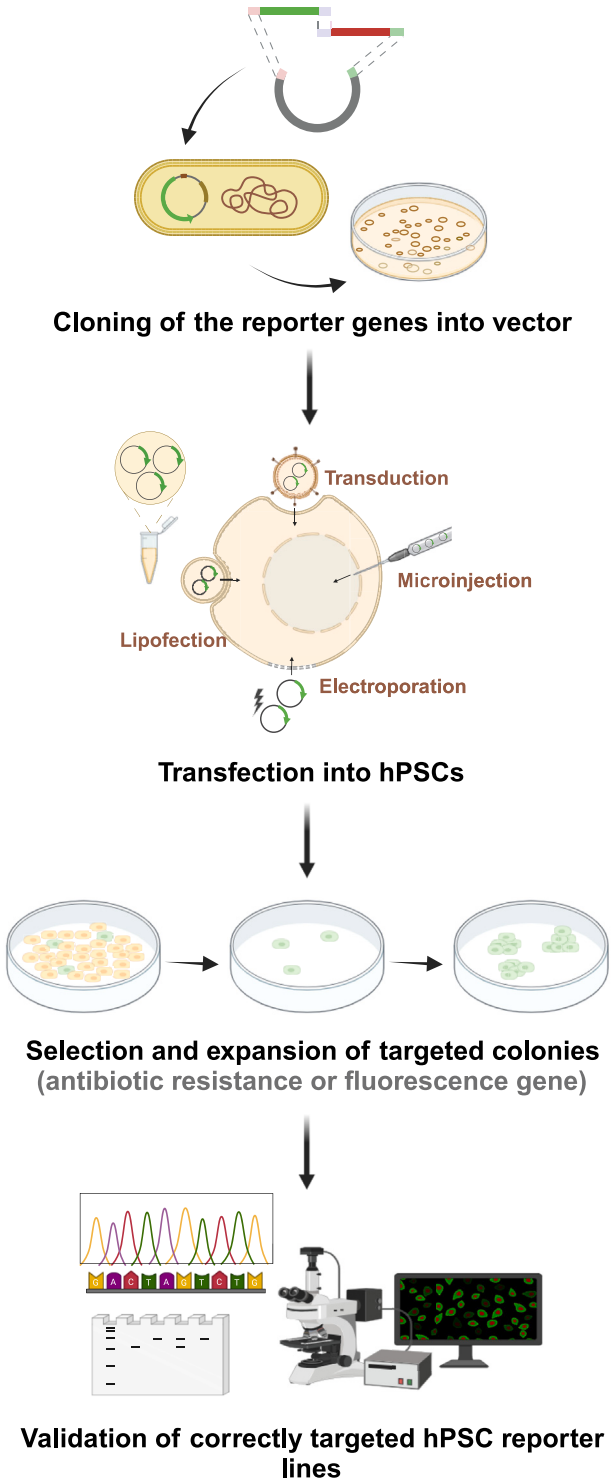


**Figure 5. Step-by-step strategy to establish an hPSC reporter cell line**

The initial step in establishing an hPSC reporter cell line involves the insertion of the reporter gene into a vector plasmid, followed by amplification. The plasmid is then inserted into hPSCs through viral transduction, chemical transfection using Lipofectamine, microinjection, or electroporation. Correctly targeted cell lines are selected using antibiotic resistance or fluorescence signals. Finally, the established cell line is validated through sequencing analysis and confirmation of reporter protein expression.

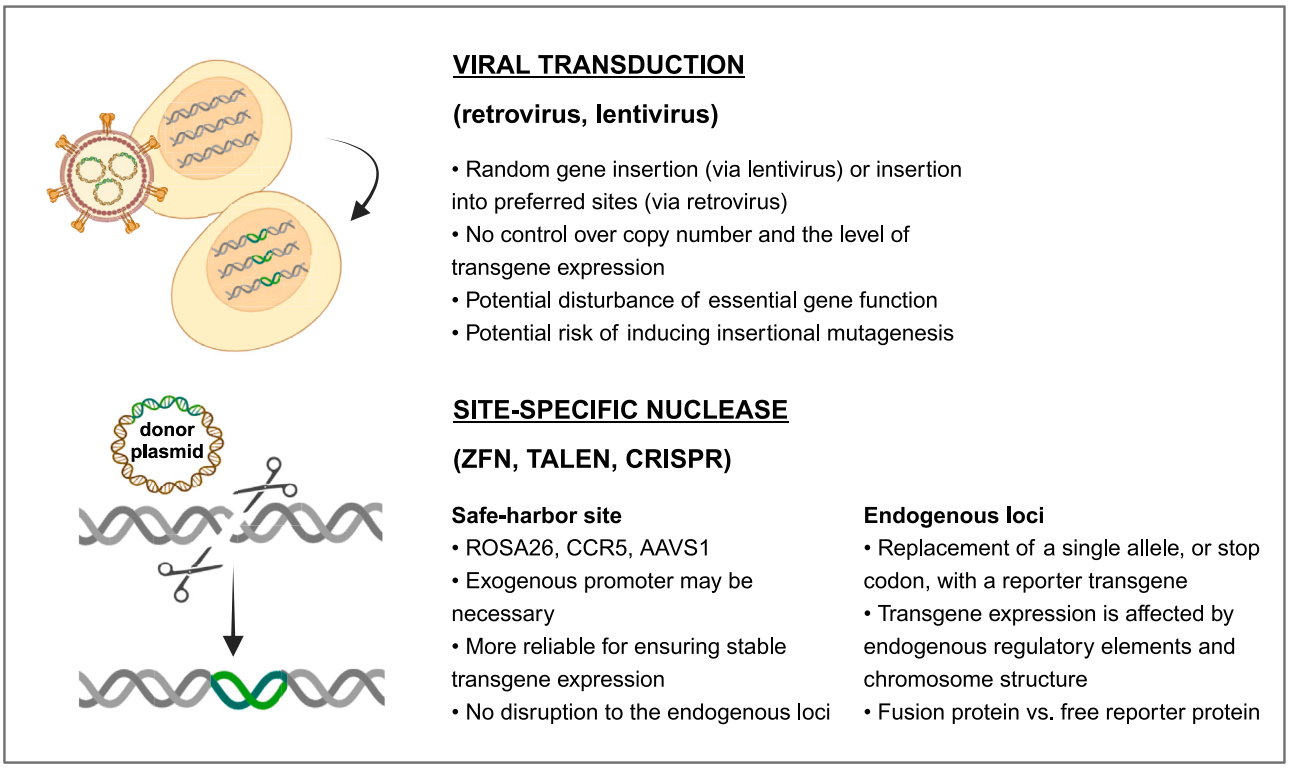


**Figure 6. Selection of gene engineering techniques for generating the hPSC reporter line**

Choosing the appropriate technique to insert a reporter transgene is crucial for achieving the desired outcomes. Among the methods available, viral transduction and site-specific nucleases are two widely used approaches for generating reporter lines from hPSCs or hPSC-derived cells. Viral transduction utilizes viral vectors to deliver genetic material efficiently into target cells, ensuring stable and high-level expression of the transgene. On the other hand, site-specific nucleases enable precise genomic modifications by introducing double-strand breaks at specific genomic locations, followed by the insertion of the reporter transgene via homology-directed repair. ZFN, zinc-finger nucleases; TALEN, transcription activator-like effector nucleases; CRISPR, clustered regularly interspaced short palindromic repeats.

system, multiple copies of reporter genes can be inserted into a single cell to amplify their expression. Lentiviral delivery has been used in many hPSC studies, such as in the establishment of a cardiomyocyte dual reporter line.[16,52] In the first round, hPSCs were transduced with lentiviruses carrying a construct of the mCherry coding sequence regulated by a specific promoter activated only in cardiomyocytes. Next, to observe the change in the cardiomyocyte action potential, a second round of lentiviral infection was performed, delivering the fluorescent voltage indicator ASAP2f.[16] It is important to note that the lack of control over the number of copies and the site of integration in retroviral- or lentiviral-derived gene editing could cause inconsistent expression patterns, silent reporter gene expression upon insertion, and increase the risk of insertional mutagenesis. Integration into critical genes or regulatory regions may interfere with normal cellular processes and gene expression.[53]

## Site-directed reporter gene integration with gene targeting

In gene targeting, a foreign gene is inserted into a specific site in the genome. This system relies on the cellular DNA damage machinery, which consists of two pathways: non-homologous end joining (NHEJ) and homology-directed repair (HDR). NHEJ repairs double-strand breaks (DSBs) by merging two broken DNA ends without a template. It is an error-prone pathway and may introduce small insertions or deletions at repair sites. HDR utilizes homologous DNA sequences, such as sister chromatids, as a template to repair DSBs.[54] Taking advantage of this process, a DNA template containing a desired insert, such as a GFP or a luciferase coding sequence flanked by two homologous regions, can be used to facilitate gene insertion. Conventional gene targeting, as has been successfully performed in mouse ES cells, is less effective in hPSCs because of the higher ratio of the NHEJ to HDR pathway in human cells.[55] Different systems, including zinc-finger nucleases, transcription activator-like effector nucleases, and CRISPR, have been developed to induce site-specific DSBs and increase the chances of HDR.[56] These systems have been used to generate reporter cell lines from hPSCs.[22,57,58]

## Targeting reporter gene into safe harbor sites

With site-specific gene targeting, the location of gene insertion is an important factor. In mice, the Rosa26 locus is considered a "safe harbor" site owing to its susceptibility to efficient gene targeting without disrupting the expression of the nearby gene.[59] A homolog of Rosa26 has been

identified in human cells and has been successfully targeted to express red fluorescent protein in hESCs.[60] The AAVS1 locus, located between exon 1 and intron 1 of *protein phosphatase 1 regulatory subunit 12C*, is possibly the most studied and well-known safe harbor site in humans and is the preferred site for adeno-associated virus integration.[61] Targeting reporter transgenes to a safe harbor site is ideal for the continuous expression of reporter genes because it allows ubiquitous transgene expression in various cell types, including hPSCs and their derivatives.[58,60] In studies that aimed to monitor the activity of a specific promoter, an artificial synthetic promoter was inserted upstream of the reporter gene.[50,62] However, other regulatory elements that may influence the original locus, such as distal enhancers and epigenetic factors, are excluded in this system. Thus, inserting a reporter transgene into the endogenous locus of the target gene may reflect its expression pattern accurately.[63]

### Reporter gene integration into the endogenous locus of a target gene

The first human rod cell reporter line was generated from hPSCs by replacing one allele of the neural retinal leucine zipper gene with an eGFP-coding sequence. With CRISPR/Cas9 technology, the *eGFP* gene was inserted adjacent to the start codon, followed by a polyA terminator and a puromycin resistance gene.[64] Because a gene copy is dysfunctional, understanding the consequence of the missing allele is crucial before choosing this strategy. Moreover, this type of reporter line monitors only promoter activity and not mRNA translation. Thus, an alternative strategy is to replace the stop codon of the coding gene with a reporter transgene, allowing the transcription and translation of the endogenous protein and the reporter. In a previous study, this strategy was implemented to generate reporters for major ectodermal lineages. A GFP-coding gene fused with the histone H2B gene was inserted into the endogenous locus of the neuroectoderm marker, *paired box 6* (*PAX6*; PAX6::H2B-GFP), and the cranial placode marker, *sine oculis homeobox homolog 1* (*SIX1*; SIX1::H2B-GFP), to trace the differentiation of the hPSC-derived ectoderm into various ectodermal progenies.[65] The histone H2B sequence was attached to allow GFP accumulation in the nucleosomes and observe chromosome dynamics during fate acquisition.[66] Similarly, adding a mitochondrial targeting sequence to the GFP-coding gene enables the translation of GFP proteins into the mitochondrial matrix. This approach has been used to study the changes in mitochondrial localization upon differentiation from hESC into ectodermal and endodermal lineages.[67]

A recent publication highlighted the use of a mitochondrial reporter to assess mitochondrial biogenesis in a model of fragile X syndrome, focusing specifically on the loss of function of fragile X messenger ribonucleoprotein 1. The study observed a reduction in both the total area and aspect ratio of mitochondria in patient-derived and knockdown hPSC lines (Figure 7A).[68] The mitochondrial aspect ratio—the length-to-width ratio—serves as an indicator of mitochondrial biogenesis. A reduced aspect ratio has been associated with increased mitochondrial fission activity (Figure 7B).[69]

### Linker peptides determine the fate of reporter protein post-translation

The choice of linker peptide determines whether the reporter protein is spliced and separated from the endogenous protein upon translation or exists as a fusion protein (Figure 8). Fusion of the target and reporter proteins ensures that both proteins share spatial and temporal patterns. However, potential challenges with a fused reporter include possible interference between protein domains, decreased activity, abnormal distribution, and metabolism of the endogenous protein.[70] Self-cleaving 2A peptides are short amino acid sequences that induce ribosome skipping during translation, enabling the expression of multiple proteins from a single mRNA transcript. Among several identified 2A peptides, the peptide derived from porcine teschovirus 1 (P2A) has shown higher cleaving efficiency than other 2A peptides (T2A, E2A, and F2A) in three human cell lines.[71] Besides linker peptides, mechanisms such as pre-mRNA splicing signals or internal ribosomal entry sites can also be incorporated to facilitate the expression of multiple genes regulated by a single promoter.[72] However, the utilization of linker peptides provides certain advantages, including their compact size (typically 18–22 amino acids) and the ability to produce proteins in precise stoichiometric ratios.[71,72]

## REPORTER GENES

Fluorescent and bioluminescent light-emitting reporter genes feature distinct characteristics and therefore serve different purposes. Understanding the advantages and disadvantages of each group is crucial in designing reporter cell lines (Figure 9). Other reporter genes such as *lacZ* and *chloramphenicol acetyltransferase* require cell lysis and radioisotope material, making them unsuitable for use in living cells.[73,74] Thus, only the fluorescent and bioluminescent reporter genes are discussed in this paper.

### Fluorescent reporter proteins

Fluorescent proteins are widely used to generate hPSC reporter lines in various fields. To generate light, a fluorescent substance absorbs high-energy light (excitation light), causing the electrons to move to an excited state. As the electron returns to the ground state, it releases the emitted light or fluorescence. This process is energy consuming; hence, the re-emitted light always has a longer wavelength than the excitation light.[75] Compared with the light produced by luminescent proteins, fluorescent proteins produce brighter signals and longer stability. With a natural half-life of approximately 26–54 h, it is an ideal reporter to track living cells or locate where a protein is expressed.[76] Additionally, genetically encoded voltage- and calcium-sensitive fluorescents have been used to visualize action potentials in hPSC-derived neurons and cardiomyocytes.[77,78]

Brainbow, a technology using multiplex fluorescence and Cre/lox recombination to label cells with an array of colors, was first developed in mice and later adapted to hPSCs.[79,80] In this system, four cassettes were inserted into the AAVS1 safe harbor site of hPSCs. Each cassette

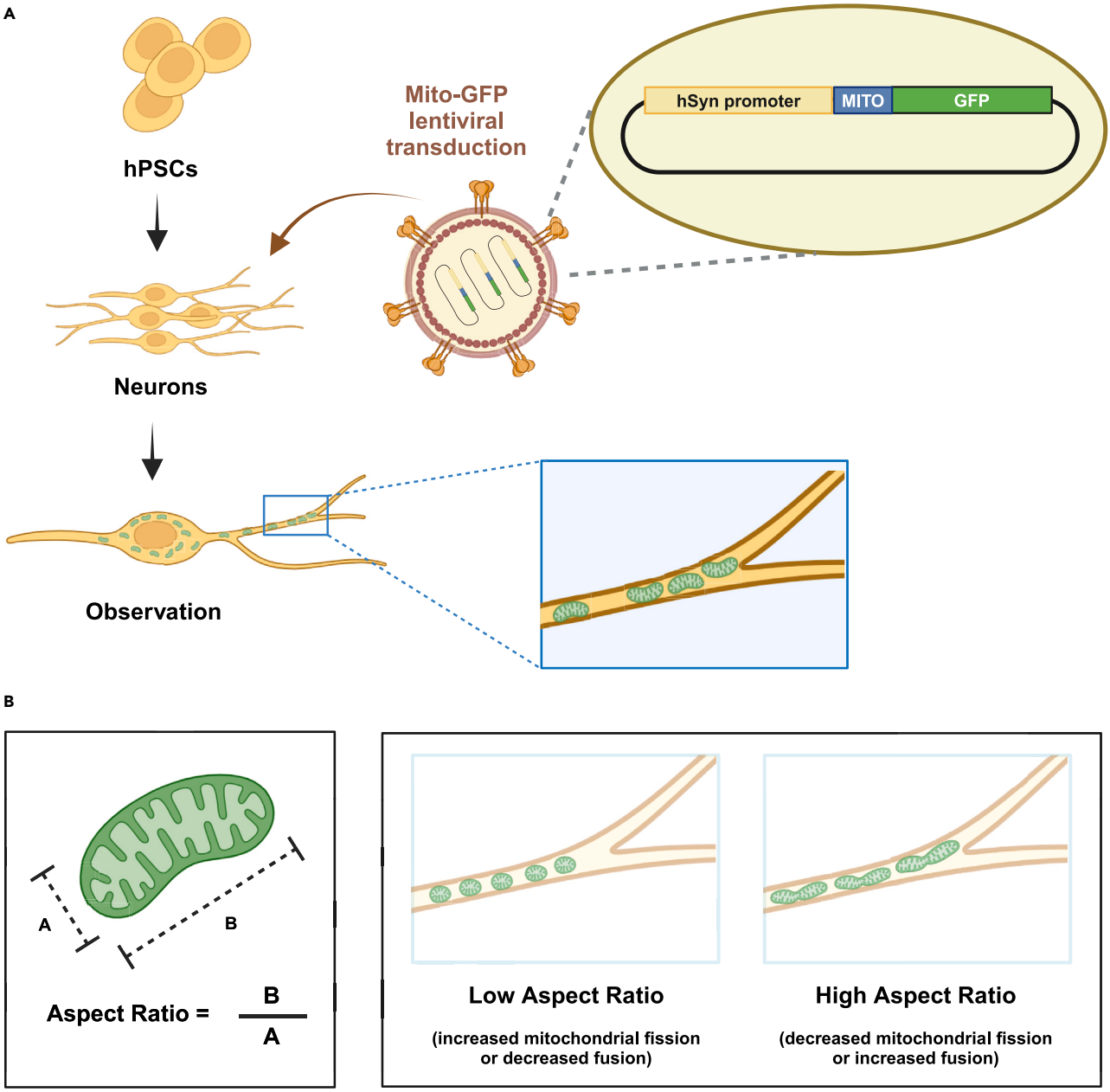


**Figure 7. Assessing mitochondrial biogenesis through the application of a mitochondrial reporter system**

(A) Incorporation of a mitochondrial targeting sequence directs proteins from the cytosol into the mitochondria, allowing *in situ* visualization of mitochondrial dynamics.

(B) Mitochondria undergo continuous fusion and fission processes critical for maintaining their integrity, quantity, and distribution within cells. The mitochondrial aspect ratio, a measure of length relative to width, provides insights into mitochondrial biogenesis by reflecting the balance of these fusion and fission events.

consisted of three unique membrane-targeted fluorescent proteins arranged in such a way that only a single fluorescent protein was permanently expressed upon Cre/lox recombination. A non-fluorescent GFP mutant was expressed in the absence of Cre. All four cassettes randomly and independently expressed one of the three fluorescent proteins, producing 18 combinations (Figure 10A). This technique allows the visual observation of cellular proliferation kinetics, clonal expansion, and cell morphology dynamics at the single-cell level (Figure 10B).[81]

Another notable system is the fluorescence resonance energy transfer (FRET), in which the excitation energy released by a fluorophore (donor) is transferred to a second fluorophore (acceptor).[82] Because the distance between both fluorophores limits this process, this system is useful for determining the distance between two molecules with high spatial resolution. Although FRET has been used by transiently expressing recombinant fluorescent proteins, to date, no stable cell line has been developed using hPSCs.[83]

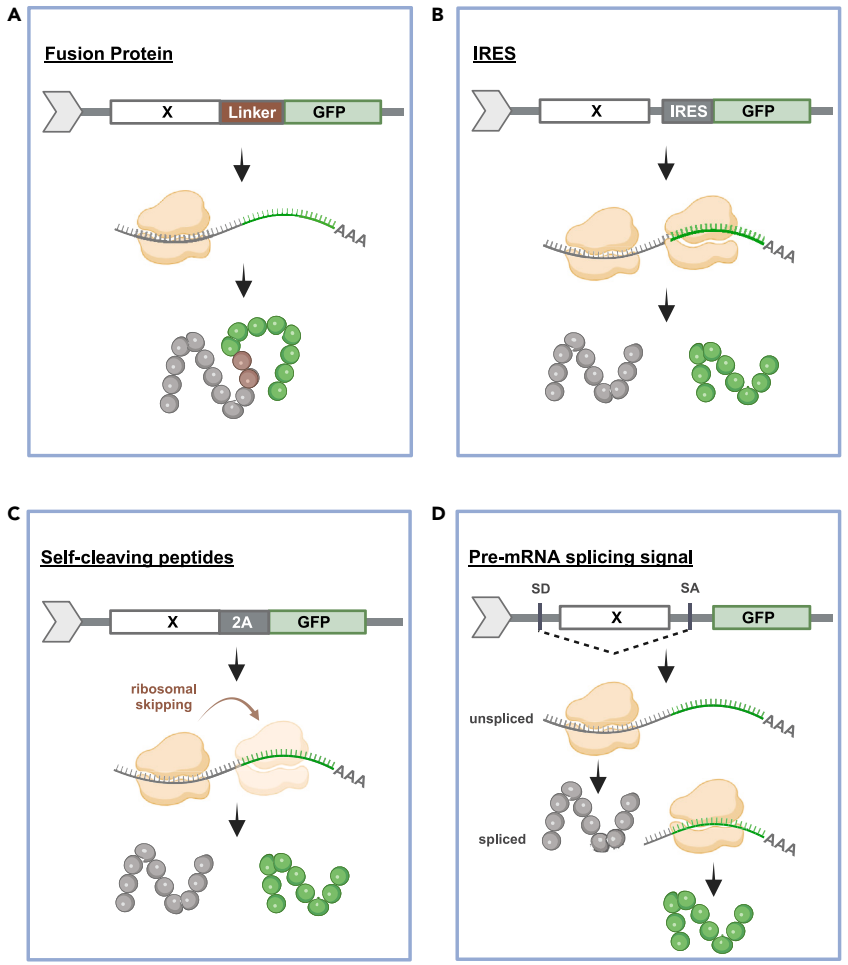


**Figure 8. Strategies for reporter gene insertion at endogenous loci and their expected outcomes**

The insertion of a reporter gene into an endogenous locus can be achieved through various methods. (A) Fusion protein generation allows the reporter gene to be directly fused with the target gene, synchronizing their spatial and temporal distribution.

(B) Addition of internal ribosome entry sites (IRES) enables the translation of the reporter gene independently from the primary coding sequence.

(C) Self-cleaving peptides facilitate the coexpression of the reporter and target genes, with the peptide ensuring their post-translational separation.

(D) Incorporation of pre-mRNA splicing signals results in two mature mRNA forms: one encoding the target protein and another encoding the reporter.

Fluorescent proteins have diverse utility, but they are not always suitable for every experimental design. For example, they may accumulate in the cytoplasm because of their long half-life, making rapid changes in gene expression difficult to observe. The bright fluorescence signal also indicates low sensitivity to slight differences in protein expression. Moreover, autofluorescence, the ability of cells to produce their own fluorescent substances, can cause a higher background when compared to luminescence, where the light is only produced by luciferase, as will be explained in the next section.

## Bioluminescent reporter proteins

Bioluminescence is the emission of light from living organisms. The chemical reaction involves the oxidation of the substrate luciferin by the enzyme luciferase, producing oxyluciferin and, importantly, light. Luciferase is expressed by many organisms and that derived from the North American firefly *Photinus pyralis* (FLuc) is the most widely used.[84] Similar to fluorescent proteins, luciferases with different characteristics are produced by different organisms. For example, the railroad worm *Phrixothrix hirtus* generates red-emitting luciferase, whereas the luciferase from the phosphobacterium *Photobacterium phosphoreum* produces blue light.[85,86] Fluc is pH-sensitive and emits yellow-green light that turns red at acidic pH.[87] Sea pansy Renilla, another commonly used luciferase, releases luminescence light with high intensity for a short period of merely 10 s, much shorter than FLuc where light is released for over 30 min.[84,88] The saltwater copepod *Gaussia princeps* and deep-sea shrimp *Oplophorus gracilirostris* produce and naturally secrete luciferase.[89,90] These variations increase the versatility of the luciferase-based reporter system.

Compared with the fluorescence signal, the light generated by luciferase is relatively dim and short-lived, making it less ideal as a cellular tracker or for studying protein localization inside the cell. However, luciferase has a shorter half-life (3–4 h) than fluorescent proteins.[91] A

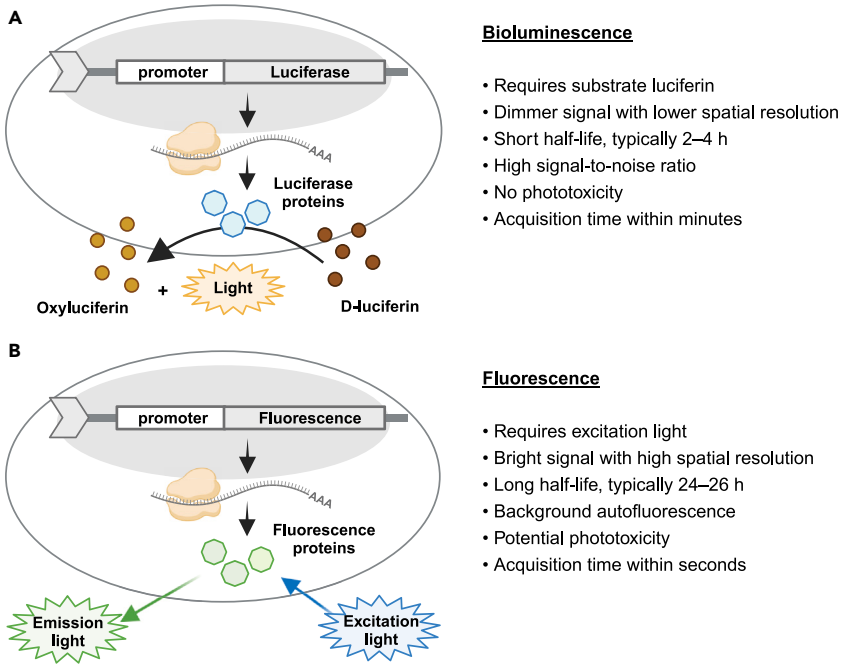


**Figure 9. Classifications of light-emitting reporter genes and their respective characteristics**

Bioluminescence and fluorescence represent two classifications of light-emitting reporter genes, each characterized by unique properties and mechanisms.

(A) Bioluminescence involves the enzymatic production of light through biochemical reactions.

(B) Fluorescence is the product of light emission from the excitation of fluorescent molecules by specific wavelengths of light.

shorter half-life is especially beneficial for observing the dynamics of gene expression, as shown in a study about the human segmentation clock and oscillatory genes that regulate the timing of epithelial somite formation during early embryonic development. In previous studies, luciferase was expressed under the control of HES7, the master regulator of oscillatory genes, to measure the period of the human segmentation clock. This study discovered that the peak of oscillations occurs every 5–6 h in humans.[92,93]

## PROMISING REPORTER SYSTEMS FOR FUTURE hPSC RESEARCH

Live cell imaging is rapidly growing. New techniques that pave the way for more accurate and detailed visualizations are continuously being developed. Some systems have been established in animal models or other human cell types but have not been used in hPSCs. For instance, HaloTag, a gene-encoded protein-tagging system that can covalently bind to synthetic ligands, can be designed to carry various molecules, including fluorescent dyes, to allow imaging of living and fixed cells.[94] A study using HeLa and HEK293T cells revealed the potential of HaloTag in measuring the rate of autophagic flux. HaloTag is resistant to the proteolytic activity of lysosomes upon binding to its ligand; thus, the degradation rate of a target protein can be observed by fusing it with HaloTag followed by treatment with fluorescent-dye-containing ligands.[95]

In addition to proteins, the transcription and localization of various RNA species with diverse roles inside living cells are equally important. A popular approach to visualize a single RNA molecule is to use RNA loops derived from bacteriophage MS2 and its natural binding proteins (MCP) that can be fused with a fluorescent protein.[96,97] However, this system has a high background signal due to unbound fluorescent proteins that move freely inside the cells. To address this issue, a previous study invented spinach RNA aptamer, an engineered short piece of RNA that can bind to a non-fluorescent fluorophore, similar to GFP, and trigger its fluorescence.[98] Since its invention, various versions of RNA aptamers that display fluorescence signals upon binding to their specific fluorophores have been continuously developed with improved stability and brightness, such as Spinach2 and Broccoli.[99,100] Studies using animal models have suggested the role of non-coding RNA species in human diseases, such as myotonic dystrophies and fragile X tremor ataxia syndrome.[101,102] Applying RNA-based reporters to hPSCs provides a human-based model to improve our understanding of such diseases in the future.

## THE INCREASING SIGNIFICANCE OF hPSC REPORTERS IN FUTURE RESEARCH AND THEIR CURRENT LIMITATIONS

The widespread development of hPSC reporter cell lines provides human-based alternatives that allow scientists to observe various phenomena in living cells with high spatial and temporal resolutions, as highlighted in this review. Recent studies have initiated a novel trend in hPSC research, particularly efforts to recreate early-stage human embryogenesis for modeling human development and associated diseases. These efforts have culminated in the generation of hPSC-derived post-implantation human embryos, comprising both embryonic epiblast-like and extra-embryonic cell lineages.[103–105] Although single-cell RNA sequencing (scRNA-seq) has facilitated the rapid identification of cellular

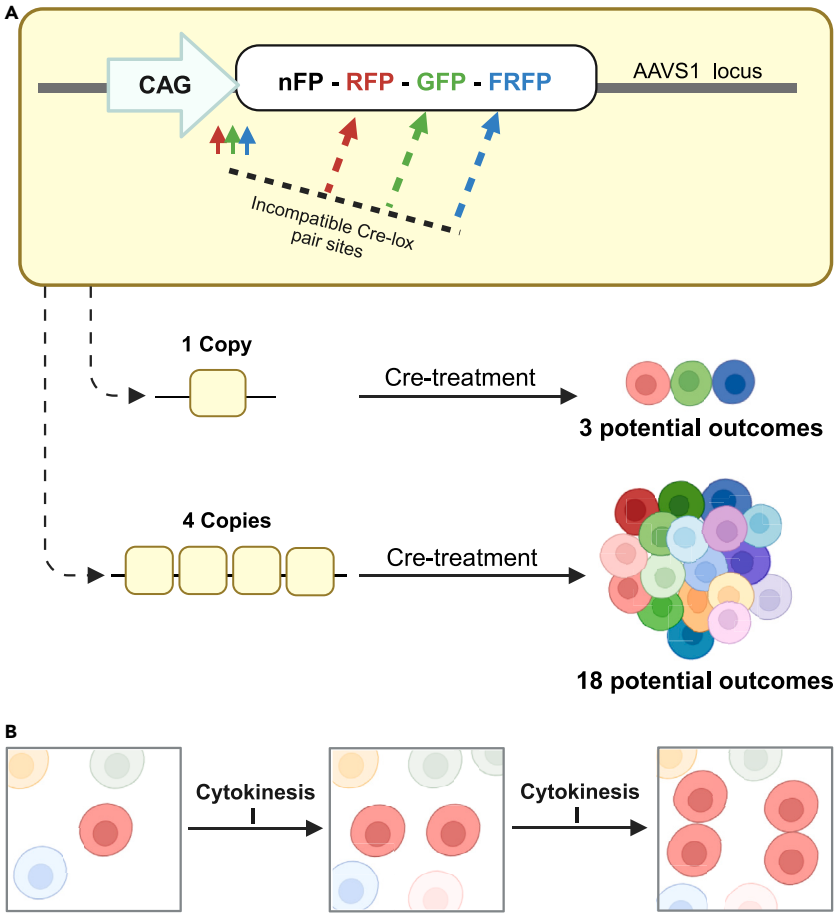


**Figure 10. Brainbow technology for multiplex fluorescence labeling of living cells**

(A) Four copies of a multicistronic cassette containing a nonfluorescent GFP mutant, RFP, GFP, and FRFP, each flanked by three pairs of incompatible Cre/lox sites, were inserted into the AAVS1 locus. Upon Cre recombinase treatment, a total of 18 possible color combinations can be expressed by individual cells.
(B) The unique fluorescent colors distinctly marked each individual cell and their descendant daughter cells, thereby enabling the tracking of cell lineage over subsequent generations.

components, it is limited in its capacity to provide spatiotemporal information essential for three-dimensional culture systems like organoids or synthetic embryos. Some studies have attempted to overcome this issue by integrating scRNA-seq with spatial transcriptomics, which maps the spatial organization and distribution of signature genes within tissue samples.[106,107] This gap can also be addressed by hPSC reporter lines, which enable time-lapse visualization of living samples.[103,108,109] With the growing enthusiasm for three-dimensional culture-based research using hPSC, understanding the topological information in organ-like structures becomes increasingly critical, highlighting the pivotal role of reporter systems in future research.

Nevertheless, the utilization of hPSC reporter lines also presents challenges that must be addressed to maximize their potential. The first is the inherent heterogeneity of hPSC lines, especially hiPSCs, which can complicate experiments because of the variability in epigenetic profiles and susceptibility to transgenic techniques.[110,111] The second is the potential for gene silencing upon differentiation into various cell types.[112–114] Despite these challenges, the knowledge and interest in molecular biology and stem cell engineering are growing rapidly. hPSC reporter cell lines are expected to continue making significant contributions to our understanding of human diseases, regenerative medicine, and drug discovery.

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## AUTHOR CONTRIBUTIONS

L.P., V.B.J., and J.S. contributed equally to conceptualizing and writing the manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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