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Applications of genetically engineered human pluripotent stem cell reporters in cardiac stem cell biology

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Abstract

The advent of human pluripotent stem cells (hPSCs) has benefited many fields, from regenerative medicine to disease modeling, with an especially profound effect in cardiac research. Coupled with other novel technologies in genome engineering, hPSCs offer a great opportunity to delineate human cardiac lineages, investigate inherited cardiovascular diseases, and assess the safety and efficacy of cell-based therapies. In this review, we provide an overview of methods for generating genetically engineered hPSC reporters and a succinct synopsis of a variety of hPSC reporters, with a particular focus on their applications in cardiac stem cell biology.

Graphical abstract

Introduction

From the first successful isolation of human embryonic stem cells (hESCs) two decades ago to the more recent breakthrough in generation of patient-specific induced pluripotent stem cells (iPSCs), human pluripotent stem cells (hPSCs) have revolutionized the ways in which scientists study developmental biology, investigate disease mechanisms, and explore new drugs in many fields [1–4]. In particular, scientists have made significant progress in

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Conflict of interest

None

cardiovascular research by developing *de novo* heart tissue as means of achieving cardiac repair [5] and understanding causes of genetic cardiac diseases using the model of “heart in a dish” [6]. Additionally, the advent of hPSCs has ushered into an exciting new era for drug discovery and safety testing [7]. These broad applications of hPSCs in the cardiovascular field have been further enhanced by the burgeoning field of genetic engineering. With the aid of efficient and precise gene targeting, genes can be readily modified to study their physiological functions and monitor their expression in hPSC-derived progeny. Hence, the marriage of hPSC biology and genetic engineering technology is enabling cardiovascular investigators to better understand the complex mechanisms of cardiac diseases and develop potential cell-based therapies. In this review, we aim to briefly summarize the genome-editing techniques commonly used for generating hPSC reporters (Table 1). Furthermore, we highlight the currently available hPSC reporters in the ever-growing tool chest and draw particular attention to the utilization of these tools to address biological questions (Figure 1).

Methods of generating genetically engineered hPSC reporters

Direct manipulation of an organism’s genes to generate transgenic animal models is an established approach to understand gene function in specific tissues. Off-the-shelf or custom-engineered gene targeting technologies are also emerging as powerful tools to generate reporter lines or correct mutations. For a detailed discussion on genome-editing in hPSCs, we refer readers to several excellent review articles [8–10]. Here, we briefly summarize the commonly used tools for generating genetically engineered hPSC reporters (Table 1).

Both random and targeted gene integration methods can be used to generate hPSC reporter lines. A reporter gene (e.g., fluorescent protein, luciferase, or antibiotic resistance gene) driven by the promoter of a gene of interest can be delivered into the hPSC genome by Bacterial artificial chromosome (BAC), lentivirus, transposons or plasmids. However, integration events are random, and the reporter may not faithfully reflect endogenous activation of the relevant gene. Moreover, the expression of inserted reporter genes can be affected by nearby gene elements or vice versa. Therefore, an effective approach to precisely insert reporter genes into the desired genomic loci is needed.

Homologous recombination-mediated gene integration allows reporter genes to share the same epigenetic conditions and chromosomal geometry with the endogenous gene of interest, allowing it to more faithfully reflect the activity of the target gene promoter. To facilitate homologous recombination, gene targeting constructs usually comprise 5’ and 3’ homology arms flanking the reporter gene and selection markers. Recombination is facilitated by introducing FLP/FRT or Cre/loxP sites into the DNA target site [11]. However, there is always a short FRT or loxP sequence left in the targeted genome, which is not “footprint” free. This limitation can be overcome by the piggyBac transposon system, which allows rapid, precise and seamless directed gene editing [12]. It should be noted that the homologous recombination is inherently inefficient. Therefore, to enhance its frequency, a double-strand break at the DNA target site is usually introduced, mediated by engineered nucleases including zinc-finger nucleases (ZFNs) [13], transcription activator-like effector nucleases (TALENs) [14], and clustered regularly interspaced short palindromic repeats

(CRISPR)/Cas9 technology [15]. CRISPR/Cas9, considered as the most efficient genome-editing approach thus far, is becoming a popular tool to generate hPSC reporter lines [16,17], as it only requires a single guide RNA and a donor vector containing the reporter gene sequence.

In summary, it is possible to insert reporter genes into any locus of the genome with innovative genome-editing tools, but several aspects of genome-editing must be considered. Extensive analysis is necessary to scrutinize the potential risk of off-target effects for nuclease, potential mutagenesis near the target site, and abnormality of karyotyping.

Applications of hPSC reporters

1. Study of cardiac lineages—*In vivo* heart development (cardiogenesis) is orchestrated by an intricate network of transcription factors that tightly regulate proliferation, differentiation and migration of specialized cells derived from multiple lineages. Cardiac developmental biologists have garnered comprehensive knowledge of cardiogenesis using lineage tracing approaches, particularly transgenic animal models bearing LacZ or fluorescent reporters at desired genetic loci [18]. Additionally, *In vitro* differentiation of hPSCs can recapitulate many cellular aspects of cardiogenesis to reveal genetic networks and signalling pathways involved in cell lineage determination and terminal differentiation [19]. Despite various cardiac differentiation approaches have been developed [20], generation of hPSC-derived cardiomyocytes (hPSC-CMs) has common sequential stages: mesoderm induction and subsequent cardiac mesoderm determination, followed by cardiac lineage specification and terminal differentiation. This developmental hierarchy of cardiac lineages is established by a set of stage-specific biomarkers (see recent review [21]). Moreover, several fluorescent hPSC reporter lines have also been generated to identify, purify, and characterize these specialized cells during this multistep cardiac differentiation process (Figure 1).

Upon mesoderm induction, hPSC-derived cells enter a transitory state, in which a multipotent population is marked by the expression of TBX transcription factor Brachyury/T (BRY), Mix Paired-Like Homeobox (MIXL1) or Kinase Insert Domain Receptor (KDR). Tracing cardiogenic lineages back to this intermediate population has led to the concept that similar to hematopoietic lineages, different cardiovascular cells such as cardiomyocytes, endothelial cells, and vascular smooth muscle cells are derived from a common cardiovascular progenitor population. Indeed, *in vitro* differentiation of hPSCs has demonstrated that BRY-GFP⁺ cells have cardiac potential [22], and KDR⁺ (low expression) cardiovascular progenitors hold the capacity to differentiate into cardiomyocytes, endothelial cells, and vascular smooth muscle cells [23]. It should also be noted that the BRY⁺/KDR⁺ (high expression) population possesses hematopoietic potential [22], suggesting that early lineage diversification is associated with the expression level of key players in the mesoderm specification. As cardiac development is a dynamic process, it is not surprising that gene expression levels display dynamic patterns to determine the cell fate. As demonstrated using a NK2 Homeobox 5 (NKX2.5, a cardiac lineage marker)-eGFP hESC reporter line, a purified eGFP⁺ population can further differentiate into eGFP⁺ cardiomyocytes and eGFP⁻ endothelial and smooth muscle cells [24]. More recently, by tagging mCherry to a cardiac

mesoderm marker, Mesoderm Posterior BHLH Transcription Factor 1 (MESP1), a MESP1^{mCherry/w}/NKX2.5^{eGFP/w} hESC dual reporter line was generated, which demonstrated a transient expression of MESP1-mCherry followed by the NKX2.5-eGFP expression [25]. This MESP1-mCherry⁺ population is defined by a distinct set of biomarkers that include PDGFR α , CD13, ROR2, and LGR4 [25,26]. Using a different hPSC reporter line of Mix Paired-Like Homeobox (MIXL1)-eGFP, Skelton et al. further corroborated that CD13 and ROR2 can be used to isolate cardiac mesoderm progenitors [27].

During cardiogenesis, commitment of nascent cardiac mesoderm progenitors towards a more specified lineage fate involves lineage segregation: one group of cardiac progenitor cells (CPCs) known as the first heart field (FHF), contributes to the left ventricle and atria, whereas a second pool of CPCs known as the second heart field (SHF) develops into right ventricle, outflow tract and atria [28]. In human fetal hearts at 11 and 18 weeks of gestation, ISL LIM Homeobox 1 (ISL1) positive cells are found in a pattern that matches the SHF-derived structure in murine fetal hearts, suggesting that ISL1 is a potential marker for the SHF [29]. Isolated ISL1⁺ cells using a ISL1-DsRed hPSC reporter are multipotent and can differentiate into cardiomyocytes, endothelial cells, and smooth muscle cells [29]. More recently, a study reported that using the NKX2.5^{eGFP/w} hESC reporter line with regulated expression of MYC (a transcription factor that plays a role in cell cycle progression), expandable hPSC-derived CPCs were generated for the first time [30]. The authors further demonstrated that by modulating FGF and BMP4 signalling pathways, these expandable hPSC-derived CPCs proceeded to the early heart field development stage characterized by unique gene expression signatures [30].

In summary, although lineage tracing mice have been widely used for understanding cardiogenesis, there are significant differences in the heart between human and mice, making it impossible for animal models to fully recapitulate the developmental process of the human heart. As alternatives, a variety of fluorescent hPSC reporter lines have been created and validated as tools more capable of faithfully recapitulating human cardiac lineages.

2. Application in cell transplantation—Because human adult cardiomyocytes lack a robust capacity to proliferate, the initial cardiomyocyte death associated with myocardial infarction can trigger cardiac remodeling and eventually lead to scarring of the heart. There has long been an urgent need to develop an effective cell-based therapy to replace these dead cardiomyocytes; to that end, hPSCs have emerged as an appealing source for cell replacement therapies for myocardial infarction. The potential and challenges of using hPSC-derived cells for cardiac repair are thoroughly reviewed elsewhere [31–33]. In this section, we focus on the application of hPSC reporters in pre-clinical studies.

To evaluate the efficacy of cell therapy, it is critical to monitor cell survival, proliferation, migration and functional integration into the host myocardium. Genetically encoded fluorescent reporters have been commonly used for long-term tracking of the grafted hPSC-CMs *in vivo* [34–36]. Stable expression of fluorescent reporter genes has advantages over transient cell labelling with fluorescent probes, because factors such as labelling efficiency, cell toxicity, and cell division-induced dilution effect may affect the interpretation of results

[37]. Interestingly, despite the low survival rate of transplanted cells, some recent studies have reported that engrafted hPSC-CMs can partially remuscularize the infarcted areas and improve myocardial function in small animal models [38–41]. Furthermore, using a genetically encoded Ca^{2+} indicator, GCaMP3, two reports showed that donor hPSC-CMs can electrically couple with host myocardium in both small animals [41] and non-human primates [42]. However, in contrast to small animals, these primates experienced ventricular arrhythmias and the cardiac function showed no significant improvement after transplantation. Taken together, fluorescent reporter lines either expressing a fluorescent protein or a Ca^{2+} indicator have shown promise in tracking hPSC-CMs and investigating functional integration of engrafted cells after transplantation. However, significant hurdles remain before these approaches can move forward toward clinical trials. One limiting factor in previous studies is the use of a heterogeneous pool of hPSC-CMs, which carries the potential risk of arrhythmias and compromises the engraftment efficiency. Therefore, transplantation of lineage-specific cardiomyocytes or ventricular cardiomyocytes purified with genetically engineered hPSC reporter lines is currently being investigated by several groups.

3. Application in purification of myocyte subtypes, disease modeling, and drug screening—Over the past few years, in parallel with development of robust protocols to efficiently differentiate hPSC-CMs (see review [20]), transgenic approaches have also been used to enrich cardiomyocytes [43–48]. However, the resulting yield is still a heterogeneous pool of nodal, atrial, and ventricular subtypes, which hinders precise disease modeling and drug testing. To overcome this hurdle, sarcolipin (SLN), atrial myosin light chain 2 (MLC-2a), and ventricular myosin light chain 2 (MLC-2v) are increasingly being used as chamber-specific markers to drive the expression of fluorescent reporter genes [44,49–51], allowing identification and purification of chamber-specific subtypes. Additionally, using a cGATA6-eGFP lentiviral vector, Zhu and colleagues were able to identify 95% of eGFP⁺ cells with pacemaker action potential (AP) morphology, suggesting that activation of GATA6 is necessary for differentiating nodal cells [52]. Moreover, by taking advantage of NKX2.5^{eGFP/w} hESCs, a directed differentiation protocol to generate nodal cells was developed [53]. These hPSC-derived nodal cells not only display the genetic signature and functional features of pacemaker cells, but also have the capacity to function as biological pacemakers when transplanted into rat hearts. More recently, the same group used NKX2.5^{eGFP/w} hESCs to identify ventricular and atrial lineages marked by CD235a and RALDH2, respectively, which diverged during the mesoderm developmental stage [54]. These studies demonstrate the broad applications of genetically engineered hPSC reporters, which not only facilitate optimization of differentiation protocols, but also help to identify novel biomarkers for cardiac lineages.

Subtype phenotyping of hPSC-CMs heavily relies on the use of single cell patch-clamp. Despite being the “gold standard”, the low throughput and invasive nature of this technique has limited its application in areas such as drug screening. Sinnecker’s group constructed a series of lentiviral vectors encoding voltage-sensitive fluorescent protein (VSFP) driven by the promoters of Short Stature Homeobox 2 (SHOX2; essential for directing sinoatrial node differentiation [55]), SLN, and MLC-2v to identify nodal, atrial and ventricular subtypes,

respectively [51]. When hiPSC-CMs from a long QT patient were transfected with these vectors, only atrial and ventricular, but not nodal cells, exhibited the prolonged AP duration by optical recording. However, when cardiomyocytes were subjected to ivabradine, a “funny” current inhibitor, only nodal cells responded. This study demonstrates the feasibility of precise disease modeling and drug screening using optical imaging in genetically modified hiPSC-CMs. Indeed, with the significant advances in the field of optogenetics, the concept of “all-optical electrophysiology” for high throughput drug screening in the cardiac field is emerging [56–59]. In combination with hiPSCs, all-optical imaging is a promising approach to address the Comprehensive *in vitro* Proarrhythmia Assay (CiPA) mandate [60].

Conclusions and future perspectives

Genetically engineered hPSC reporters are a powerful tool with far-reaching applications in cardiovascular research and medicine. In this review, we summarized recent advances in studying cardiac lineages, cardiac regenerative medicine, precise disease modeling, and drug testing using hPSC reporters. Cardiogenesis is a complex process in which multiple specialized cells interact and migrate, leading to a 3D structure. However, the current lineage study model using hPSC reporters is predominantly based on a 2D monolayer, which does not fully represent cardiogenesis *in vivo*. Therefore, it is critical to build a more complex 3D organoid heart using hPSC reporters to gain a deeper understanding of cardiogenesis. In addition, multi-color reporter constructs and mosaic analysis, which have shed light on the complex development of the intestine and cerebellar cortex [61], may be useful in future studies of cardiogenesis. Multi-color hPSC reporters are expected to be available in the near future to monitor the activation state of intracellular signalling pathways, cell turnover, and motility of different cell types derived either from the same or different lineages. Furthermore, with recent advances in microfluidics, single-cell analysis paired with hPSC reporters will be able to reveal cell heterogeneity and build a precise roadmap of lineage hierarchy in cardiogenesis.

A better understanding of human cardiac lineages is also essential for identifying and isolating suitable hPSC-CPCs for regenerative medicine. Transplantation of hPSC-CPCs may have advantages over hPSC-CMs, as CPCs are multipotent and able to differentiate into all types of cardiac cells, which is essential for maintaining proper function of engraftments. Moreover, using functional reporters such as genetically encoded Ca^{2+} indicators or voltage sensors would not only allow the donor cells to be tracked, but may also improve our ability to study electrical coupling between donor and host cells. To these ends, optical sensors with a favourable combination of brightness, dynamic range, and speed are necessary.

A central goal of Precision Medicine Initiative is to make precise disease modeling and drug testing a reality. Recently, hPSC reporters have played an important role in developing directed differentiation protocols that make scalable production of hPSC-derived myocyte subtypes possible, taking us closer to precise disease modeling and drug testing. The future of applying hPSC reporters in different disciplines of biomedical research is bright. Combined with other novel technologies (e.g., single cell analysis), hPSC reporters will have even broader applications and contribute to our growing insights into the mysteries and complexities of cardiac stem cell biology.

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- Human pluripotent stem cells (hPSCs) revolutionize cardiac research.
- HPSC reporters are commonly generated by lentivirus and homologous recombination.
- A variety of hPSC reporters are used to delineate cardiac lineages *in vitro*.
- HPSC reporters facilitate to track engraftments in animals.
- HPSC reporters provide a platform for precise disease modeling and drug screening.

Figure 1. Schematic summary of applications of genetically engineered hPSC reporters in the cardiac research

A variety of hPSC reporters have been generated either using virus-mediated or homologous recombination-mediated methods. These hPSC reporters have been used for delineation of human cardiac lineages *in vitro*, development of directed differentiation protocol for myocyte subtypes, tracking of engrafted hPSC-CMs, and precise disease modeling and drug testing. Dashed arrow indicates the potential application. hPSC: human pluripotent stem cell; BRY: Brachyury; MIXL1: Mix Paired-Like Homeobox; MESP1: Mesoderm Posterior BHLH Transcription Factor 1; NKX2.5: NK2 Homeobox 5; ISL1: ISL LIM Homeobox 1; SHOX2: Short Stature Homeobox 2; cGATA6: chicken GATA Binding Protein 6; MLC2a:

Myosin Light Chain 2 atrial isoform; MLC2v: Myosin Light Chain 2 ventricular isoform; SLN: Sarcoplipin; ACTB: Actin Beta; NCX: Na⁺/Ca²⁺ Exchanger; αMHC: α-Myosin Heavy Chain; GFP: green fluorescent protein; eGFP: enhanced green fluorescent protein; VSFP: voltage sensitive fluorescent protein; GCaMP3: GFP-based Ca²⁺ indicator.

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Table 1

Summary of methods for genetic engineering and typical examples of application in cardiac field.

	Targeting methods	Overall Advantages	Overall Disadvantages	Applications	
Random integration	Bacterial artificial chromosome (BAC)	Rapid and easy operation	• Random integration	1	Cardiac lineage studies [22,25,27,29]
	Lentivirus		• Risk of silencing		
	Transposons		• No control of gene expression level		
	Plasmids			2	Functional integration studies [41,42,48]
Targeted integration	Zinc-finger nucleases (ZFNs)	• Endogenous control of reporter gene expression	• Risk of off-target	3	Cardiomyocyte subtype studies [51–54]
	Transcription activator-like effector nucleases (TALENs)		• Locus dependent targeting efficiency		
	Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9	• Precise targeting • Higher efficiency			