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

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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.

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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 [1,2••,3,4]. In particular, scientists have made significant progress in 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•,9,10]. 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

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) Lentivirus Transposons Plasmids	Rapid and easy operation	• Random integration • Risk of silencing • No control of gene expression level	1. Cardiac lineage studies [22,25•,27,29] 2. Functional integration studies [41,42,48]
Targeted integration	Zinc-finger nucleases (ZFNs) Transcription activator-like effector nucleases (TALENs) Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9	• Endogenous control of reporter gene expression • Precise targeting • Higher efficiency	• Risk of off-target • Locus dependent targeting efficiency	3. Cardiomyocyte subtype studies [51•,52,53••,54••]

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

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