CHAPTER 2

Fluorescent Reporters In Human Pluripotent Stem Cells: Contributions To Cardiac Differentiation And Their Applications In Cardiac Disease And Toxicity

Sabine C. Den Hartogh[1], Robert Passier[1,2]

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1. Department of Anatomy and Embryology, Leiden University Medical Centre, Leiden Postal zone S-1-P, P.O. Box 9600, 2300 RC Leiden, The Netherlands
2. Department of Applied Stem cell Technologies. MIRA Institute for Biomedical Technology and Technical Medicine. University of Twente, P.O.Box 217, Enschede, The Netherlands

ABSTRACT

In the last decade, since the first report of induced pluripotent stem cells, the stem cell field has made remarkable progress in differentiation to specialized cell-types of various tissues and organs, including the heart. Cardiac lineage- and tissue-specific human pluripotent stem cell (hPSC) reporter lines have been valuable for the identification, selection and expansion of cardiac progenitor cells and their derivatives, and for our current understanding of the underlying molecular mechanisms. In order to further advance the use of hPSCs in the fields of regenerative medicine, disease modeling and preclinical drug development in cardiovascular research, it is crucial to identify functionally distinct cardiac subtypes and to study their biological signalling events and functional aspects in healthy and diseased conditions. In this review, we discuss the various strategies that have been followed to generate and study fluorescent reporter lines in human pluripotent stem cells and provide insights how these reporter lines contribute to a better understanding and improvement of cell-based therapies and pre-clinical drug and toxicity screenings in the cardiac field.

INTRODUCTION

Human pluripotent stem cells (hPSCs), i.e. embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), can be maintained in culture indefinitely and since they have the potential to differentiate into any cell type of the human body, these cells offer an unlimited source of differentiated cell types, including cardiomyocytes (CMs). The promise of hPSC-derived CMs for treatment of heart disease has grown rapidly since the ability to reprogram somatic cells into iPSCs, discovered for the first time by Yamanaka et al. in 2006 [1]. IPSCs are very similar to embryonic stem cells (ESCs), but are of a less ethical concern, as they do not require donated embryos for derivation. Further, they can be generated from patients with genetic disease to create *in vitro* human disease models. In recent years, the differentiation efficiency from hPSCs to cardiac cells has increased considerately, yielding 60-90% cardiomyocyte cultures[2]. Efficient directed differentiation of CMs from hPSCs is of high interest for multiple fields of research, including cardiogenesis, *in vitro* disease modeling, drug discovery, safety pharmacology and regenerative medicine. Fluorescent reporter lines offer the opportunity to monitor cell subtype specification and to investigate underlying molecular mechanisms during differentiation of hPSCs towards cardiac cells, and to track cells in cell-based therapies. In order to generate fluorescent reporter lines, hPSCs need to be genetically modified, which can be performed through different approaches. A variety of fluorescent proteins that cover a broad range of the wavelength spectrum, including eGFP, mCherry and mVenus, enable imaging and tracking of specific cell populations and mapping their cell fate. Here, we will summarize the different fluorescent reporter lines that have been described so far, how these were generated, and how they have contributed to our current knowledge on cardiac progenitors segregation, proliferation, and subtype specification. Further, we describe their importance for applications in regenerative medicine (e.g. replacing damaged or non-viable heart tissue) and further advancing *in vitro* systems for disease modeling and drug discovery and safety testing **(Figure 1)**.

1 Methods for Generating PSC Reporter Lines

For selection and purification of specific cell types, non-genetic approaches such as metabolic selection or sorting using cell type-specific antibodies are preferred. However, these approaches are not always available or fail to yield sufficient purity, and therefore, the use of cell-type specific fluorescent reporter lines offers an excellent alternative. Generation of these reporter lines require genetic engineering in hPSCs, for which several successful approaches have been described. Each genetic engineering approach knows its own advantages and disadvantages, which we will discuss here briefly **(Table 1)**. More specific details can be found elsewhere[3-5].

1.1 Promoter-driven Reporter Lines

Introducing exogenous regulatory fragments that are coupled to a fluorescent reporter gene can generate cell-specific promoter reporter lines. These elements can be introduced randomly into the genome through viral-mediated transduction[6], electroporation, nucleofection, or chemical-based transfection. However, important shortcomings exist in using these methods. First, integration events are random and there is no control over copy number, site of integration, and gene expression level. Moreover, chromatin dynamics and epigenetic regulators may change upon lineage differentiation, resulting in the chance of transgene silencing disabling gene reporter expression[7-9]. Second, endogenous regulatory elements may interfere with the incorporated promoter/enhancer elements, so that the fluorescent reporter may not faithfully recapitulate endogenous activation of the target gene during differentiation. The use of strong insulator elements flanking the enhancer and its reporter gene could be a way to overcome transgene silencing[9, 10]. Moreover, endogenous elements themselves could be influenced by the integration of new regulatory elements, which may affect the differentiation process, cellular behavior and function. Several loci have been described to be ubiquitously expressed in hESCs and progeny, including sequences highly homologous to the mouse Rosa26 locus and the AAVS1 locus, which could be targeted through homologous recombination[11-13]. However, it should be noted that transgene silencing can be lineage-specific and the effect of gene expression following gene insertion in these loci have not been described for all mature cell-types. Third, these strategies are always based on restricted regulatory elements, often based on conserved elements between species. However, gene regulation is complex and can be regulated through combinatorial distal and proximal elements that control the precise expression pattern of a gene during development[14], and therefore detailed knowledge of specific regulatory elements and their effect on gene regulation is required. The best way to overcome these limitations is the development of a knock-in PSC line.

1.2 Targeted Genome Editing

With targeted genome editing, a reporter gene is inserted into a specific site at the gene of interest through homologous recombination[15]. In recent studies using cardiac reporter lines in hESCs, sequences following the translation start codon of the gene of interest were replaced in frame with the coding sequence of a fluorescent reporter gene, in the presence of adjacent to a positive selection cassette. A targeting vector containing these sequences are flanked by sequences homologous to the genomic regions at the 5' and 3' ends of the targeting site, required for homologous recombination[16, 17]. Sequential excision of the selection cassette is recommended in order to prevent interfering regulatory events at the targeted site[18]. One major disadvantages of this strategy is the generation of haploinsufficient hPSC lines, since in one allele the targeted gene of interest has been replaced with a fluorescent encoding protein. Therefore, it is important to have additional information on whether heterozygote expression of the target gene may affect differentiation and cellular functions. For example, studies in knockout mice or comparable strategies in other species may provide this information. Alternatively, an internal ribosomal entry site (IRES) or viral 2A peptide may be incorporated in the targeting construct in order to allow expression and translation of both the endogenous as well as the reporter genes[19]. However, in case of the use of IRES, it has been shown by many researchers that transcription of the second gene is less efficient than the first, which also depends on the strength of the endogenous promoter[20, 21]. Viral 2A could be a superior alternative to the widely used IRES. By using a short sequence, multiple proteins can be co-expressed from a single mRNA. However, the disadvantage is that due to ribosomal cleavage of the 2A sequence, several amino acids are added to the C terminus of the endogenous protein, and one amino acid is added to the N-terminus of the reporter protein, which may have an effect on the function or turnover of proteins[19, 22].

1.3 The Promise of Engineered Nucleases

Other site-specific genetic engineering methods that have raised interest in recent years are engineered nucleases, including zinc-finger nucleases (ZFNs)[23, 24], transcription activator-like effector nucleases (TALENS), or the most recently described clustered regularly interspaced short palindromic repeats (CRISPR) technology[4]. Important considerations to take into account when choosing a specific technology, include the size of the insertion sequence, the risk for off-target modifications[25], and the prospected efficiency of the targeting. CRISPR technology has shown high efficiency (up to 51-79% in hPSCs[26] and reliability (low off target effects) in site-specific amino acid replacement, and is considered a promising technique for disease modeling or for genetic repair. Moreover, compared to ZFNs or TALENs, it only requires the generation of a single guidance RNA (sgRNA) molecule, CAS nuclease mRNA, and a donor vector in case of an insertion, which makes it much less labor-intensive[5]. Jaenisch et al. have shown that the use of CRISPR/Cas9 can be successfully used for the efficient generation of fluorescent gene reporters in mice[27]. Here, Cas9 mRNA, sgRNA, and a donor vector containing a 3 Kb transgene cassette were injected into zygotes. From the blastocyst stage-derived mESCs, 33% were correctly targeted. However, limitations still exist and may be different in hPSCs; here, the insertion of large DNA constructs into the genome has not been described yet. For CRISPR/CAS9 technology, limitations include the variance in targeting efficiency, which depends on the size of the epitope or fluorescent tag, the amount of targeting vector to be injected, cell type, cell cycle phase[28], and the target locus, and will require model-specific optimization. More specific details are reviewed elsewhere[4, 29].

In summary, several aspects are important to take into account for generating fluorescent reporter lines, such as the amount of time and labor it takes to generate a line, the efficiency of targeting, the choice for a replacement or a fusion strategy, random gene insertions from lentiviral particles, the risk of silencing, the risk for off-target effects from nucleases, and the size of the DNA construct (a fluorescent protein and/or selection cassette). An overview of the genetic engineering methods that have been used to introduce reporter constructs in PSCs for cardiac development/differentiation and transplantation studies can be found in **Table 1**. So far, most cardiac fluorescent reporter PSCs have been generated through the use of lentiviral or homologous recombination approach, although it is expected that future studies will increasingly report generation of fluorescent reporter lines through the use of engineered nucleases[4, 28].

2.1 Fluorescent Reporters to Study Segregation of Early Cardiac Progenitors

In Vivo Cardiac Development

Since differentiation of hPSCs to the cardiac lineage recapitulate the early steps of *in vivo* heart development[30], a thorough understanding of the molecular cues and signaling pathways of this developmental process is essential. The earliest precursors for heart-forming cells are derived upon ingression of epiblast cells through the vertebrate primitive streak. During this event, called gastrulation, cells undergo epithelial-to-mesenchymal-transition (EMT) and form mesendoderm. They are marked by activation of transcription factors, including T-box transcription factor brachyury T (BryT) and homeodomain transcription factor MIXL1[31-33]. Another transcription factor, Eomesodermin (EOMES) is restricted to the anterior region of the primitive streak[34], and together with BryT they act upstream of bHLH transcription factor MESP1. In combination with low Nodal signaling, this results in the transition from a mesendodermal stage to a more defined pre-cardiac subpopulation, marked by MESP1[35-37]. Almost all cells of the final developing heart, but also hematopoietic and skeletal myogenic lineages, are derived from MESP1-expressing early progenitors[38, 39]. Recently, a more restricted cardiac progenitor cell population, derived from Mesp1, and marked by Smarcd3 was described[40]. However, information

on the segregation and existence of intermediate cardiac progenitors is sparse. Based on recent clonal analysis studies in mice, early cardiovascular lineage specification is described to occur prior to, or during, Mesp1 expression[40, 41], which distinguishes the so-called first heart field (FHF, which forms the initial heart tube, and later the left ventricle (LV) and some of the right ventricle and atria) from the second heart field (SHF, marked by ISL1 expression, which forms the right ventricle (RV), atria, and outflow tract[42]. FHF progenitors are located within the so-called cardiac crescent, and following migration and fusion at the midline, a primitive heart tube is formed. SHF cells are located medially and posteriorly to the cardiac crescent and contribute to the formation of the heart at a later stage. In the developing human heart, ISL1-expressing progenitors are described to differentiate to different downstream multipotent heart progenitors that contribute to SHF-derivatives[43, 44]. In contrast, the highly conserved cardiac transcription factor NKX2-5 is first expressed in the cardiac crescent, but later as well in SHF-derived cardiac cells. Expression of NKX2-5 is maintained throughout the developing and adult heart[45].

In Vitro Cardiac Reporter Lines

A genetically modified mouse ES cell line, in which Mesp1 regulatory regions were coupled to a GFP reporter, showed the ability to isolate and study early Mesp1-GFP cells upon cardiac differentiation[46]. They identified the cell surface markers CXCR4, Flk1, and PDGFRa to be specifically expressed in Mesp1-progenitors in mice. Single cell clone analysis demonstrated the heterogeneous expression of early cardiac genes, including Isl1, indicating early segregation of Mesp1 progenitors into different downstream multi-potent cardiac progenitors. This was confirmed by an *in vivo* study in mouse, where they showed early segregation of the FHF and SHF, during MESP1 expression[41]. Recently, we characterized for the first time human pre-cardiac MESP1-progenitors differentiating to the cardiac lineage[16]. We performed targeting of the genomic locus of MESP1 through homologous recombination by replacing one allele with a DNA sequence encoding for the fluorescent protein mCherry. Microarray analysis of MESP1-mCherry positive cells largely revealed similar transcriptional characteristics to those in mice. However, a clear difference between mouse and human cardiac cell differentiations is that human MESP1 progenitors appear to have a more demarcated mesodermal character, as only a small subset of early cardiac genes is expressed in this population, including GATA4, MYL4, and MYL7. Interestingly, we also identified the absence of surface markers that were previously identified on mouse Mesp1-GFP progenitors[46]. Human MESP1 progenitors do highly express PDGFRa protein, but not CXCR4, and only a low percentage expresses KDR, indicating the presence of a PDGFRa+KDR- cardiac progenitor population, besides the previously described human KDR+PDGFR+ cardiac progenitor[47].

As described above, the LIM-homeodomain transcription factor Isl1 is enriched in the secondary heart field[42]. Mouse multipotent Isl1+ cardiac progenitors contribute to endothelial, endocardial, smooth muscle, the conduction system, right ventricular, and atrial cardiomyocytes[43, 48]. By generating an *in vitro* Isl1-Cre Knock-in DsRed reporter line in hESCs for lineage tracing construct it was found that human ISL1+ progenitors can give rise to cardiomyocytes (cTnT), endothelial cells (PECAM1), and smooth muscle cells (SM-MHC)[44]. Furthermore, this study, and several others, showed expansion of Isl1+ progenitors under activation of the Wnt/B-catenin-pathway[44, 49, 50]. More insight in the ability to maintain and expand cardiac progenitors may provide an unlimited source of cells committed to form cardiovascular cells.

As mentioned previously, NKX2-5 is a key cardiac transcription factor and is expressed in heart precursor cells in the FHF, which are committed to the cardiac lineage[51]. Elliott et al. designed a NKX2-5 fluorescent reporter line in hESCs, by targeting a DNA sequence encoding for eGFP into the NKX2-5 genomic locus through homologous recombination[17]. Since NKX2-5 is activated in cardiac progenitors and expression is maintained in functional cardiomyocytes at later stages, this reporter line is very suitable for studying the effect of small molecules and growth factors during cardiac lineage differentiation from hESCs. Indeed, following this approach, a more efficient, robust and defined differentiation protocol was attained. In addition, Birket et al. recently showed in a MYC-inducible system expansion of hESC-derived NKX2-5-eGFP+ progenitors for many passages through IGF-1 and hedgehog signaling, from which different cardiac subpopulations, including nodal-like cells, could be obtained[52]. Furthermore, using antibody arrays and gene-expression arrays, a cell surface protein signal regulatory protein (SIRPA/CD172a) was identified as a marker for early cardiac progenitor cells. Upon further differentiation, co-expression of SIRPA with VCAM1 was identified to specifically enrich for cardiomyocytes[17, 53]. Interestingly, SIRPA is not expressed in the developing mouse heart, in contrast to the human heart, which highlights the need to define markers in the human system. The previously described MESP1-mCherry targeting was performed in this NKX2-5-eGFP hESC line, yielding a dual cardiac reporter line **(Figure 2)**[16], which facilitates studying molecular pathways that are involved in the early switch from hESCs to pre-cardiac mesoderm, and its subsequent differentiation towards the cardiac lineage and specification into different cardiac subtypes. This could also provide opportunities for monitoring cardiac-committed progenitors (MESP1+) to NKX2-5+ cardiomyocytes following transplantation in infarcted hearts (see section 3.1. Fluorescent Reporters to Track Cells after Transplantation).

2.2 Fluorescent Reporters to Study Cardiomyocyte Derivation

The major cell types of the heart include ventricular and atrial cardiomyocytes, cardiac conduction cells, vascular smooth muscle cells, endothelial cells (myocardium and endocardium), and cardiac fibroblasts. Both for cardiac regeneration therapies and biomedical applications it is important to establish defined scalable culture systems or tissue constructs consisting either of pure cardiac cell populations or a well-balanced and controlled mixture of cardiac cells resembling the composition and architecture of the human heart.

However, current cardiac differentiation protocols result in a mixture of cardiac and non-cardiac cell-types. Approaches to purify cell populations using fluorescent reporter lines under the control of cardiac- and/or subtype-specific promoters have been first performed in mESCs[54-57]. In hESC an alpha-MHC or NCX-1 promoter, coupled to either a fluorescent protein (eGFP, mCherry), or the antibiotioc resistance gene, puromycin has been introduced into the genome [6, 58-60]. In the study of Kita-Matsuo et al., α-MHC positive CMs were selected in the presence of puromycin for 36 hours (day 12 to day 13.5), which yielded 92-96% pure CMs (based on quantifications of cardiac Troponin I immunostainings)[6]. Differentiation of NCX1-eGFP hESCs resulted in a maximum of ±50% of NCX-1-eGFP+ CMs, highly enriched for early and late cardiac genes, including NKX2-5, TBX5, cardiac troponin T, MYH6, and MYH7[59]. However, as alpha-MHC and NCX-1 are both expressed in all types of CMs, this results in a mixture of nodal, atrial-, and ventricular-like CMs. However, Both NCX-1 (Na+/Ca2+exchanger) and alpha-MHC are widely expressed in all subtypes, resulting in a mixture of nodal, atrial-, and ventricular-like CMs [6, 59].

2.2.1 Ventricular Cardiomyocytes

In order to select ventricular cardiomyocytes, promoter/enhancer elements of ventricular-specific (or –enriched) genes, such as ventricular myosin light chain 2v (MLC2V) or IRX4 could be appropriate candidates for generating subtype-specific reporter lines[61]. MLC2V expression is restricted to the ventricular segment of the developing human heart, followed by expressed in the entire ventricular compartment in the adult heart[62]. It is noteworthy that different cis-acting regulatory elements may be responsible for MLC2V expression in either the left or right ventricle, indicating differences in the regulation of their derivation and the possibility to identify and purify left (predominantly FHF- derived) or right ventricular (predominantly SHF-derived) cardiomyocytes[45, 63]. Huber and colleagues generated hESCs stable lines, using lentiviral vectors that expressed fluorescent protein eGFP under the transcriptional control of a highly conserved 250 bp MLC2V promoter element[64]. This enabled a highly enriched population of ventricular-like cells, purified by FACS[65]. It should be noted that the used promoter is dominantly active in right ventricular CMs[64]. Alternatively, the use of a drug resistance gene under the control of a ventricular-specific promoter may be another strategy. A disadvantage of selecting ventricular CMs through the MLC2V promoter is its low expression level in early stage hPSC-CMs. Substantial MLC2V expression levels are displayed only after long-term cultures for approximately a month, which is correlated to a higher degree of maturation [66]. Interestingly, most CM differentiation protocols that exist show a preferred differentiation to the ventricular lineage. However, it is not known whether these ventricular cells are derived from the same or from different cardiac progenitor cells, and whether it is important for development of directed differentiation protocols. The use of dual or multi-reporter hPSC lines may provide better insights in this matter.

2.2.2 Atrial Cardiomyocytes

Factors that determine fate specification of atrial cardiomyocytes are largely unknown[67], although several atrial-specific or –enriched genes have been identified previously in different species. For example, myosin light chain 2A (MLC2A) is initially expressed throughout the tubular heart in the mouse embryo, and becomes only confined to the atrial segment during chamber formation[45]. This is in contrast with human cardiac development, where MLC2A transcript was found in both atria and ventricles at even later stages of development[62], signifying the importance to understand the molecular mechanisms that are involved at the different stages of human cardiac development in order to serve as a blueprint for directed step-wise cardiac differentiation of hPSCs. Recently, the orphan nuclear receptor COUPTFII was identified as an important regulator of atrial development in mice[67]. Upon *in vitro* cardiac differentiation from hPSCs in the presence of retinoic acid, we recently demonstrated that both COUPTFI and COUPTFII are involved in atrial cardiomyocyte derivation[68]. Moreover, both transcription factors were highly enriched in human atria at RNA and protein levels during cardiac development and both genes may represent interesting candidates for generating atrial-specific reporter hPSC lines. However, expression of COUPTFII can also be found in venous/lymphatic endothelium, vascular smooth muscle cells, the endocardium, and the epicardium[69].

2.2.3 Nodal-like Cardiomyocytes

For understanding molecular pathways leading to differentiation of nodal-like hESC-CMs and to allow their isolation, promoter-enhancer elements of several genes involved in the development of nodal tissue have been used so far. Previously, *in vivo* studies in mice showed selective activation of a chicken GATA6 (cGATA6) proximal promoter-enhancer element in the atrioventricular (AV) node and the bundle of His of the adult mouse heart[70]. Zhu and colleagues coupled this cGATA6 regulatory element to eGFP in a lentiviral construct and subsequently transduced hESCs prior to differentiation (with a transduction efficiency of only ~50%). By adjusting cardiac differentiation via inhibition of Neuregulin-1/ErbB signaling, an increased proportion of nodal-like CMs were identified[71]. cGATA6-eGFP positive cells exhibited action potential characteristics of a nodal-phenotype, indicated by the increased expression of HCN4, responsible for the funny current If, and transcription factor TBX3, important for *in vivo* specification of nodal tissue. Studies in mouse tested Hcn4 promoter/enhancer elements to investigate the derivation of nodal-like CMs, *in vitro* [72, 73]. Differentiating Hcn4-promoter-GFP mESCs resulted in only a small percentage of Hcn4 positive cells (±1%) exhibited a pacemaker-like action potential after fluorescent-based isolation[72]. Direct reprogramming of HCN4-GFP fibroblasts, using a combination of four transcription factors (Tbx3, Tbx5, Gata6, and Rxra), robustly activated HCN4p-GFP expressing cells, but without displaying electrical activity and no expression of sarcomeric proteins[73]. Alternative approaches for directed differentiation including the use of RNA molecules, such as modified mRNA, miRNAs, or lncRNAs are currently evaluated. Important

to note is that at the cardiac crescent stage, Hcn4 is also expressed in early FHF progenitors and immature cardiomyocytes[73-76], and only becomes confined to components of the conduction system at later stages of development[77], which is of critical importance to realize when studying the derivation of human conduction cells.

3 The Promises and Challenges of hPSC-derived CMs for Cellular Therapy

Initially, researchers focused on the potential of hPSC-derived CMs for cell replacement therapy in experimental models of heart disease, such as myocardial infarction[78, 79]. In order to restore heart function and maintain long-term beneficial effects, sufficient transplanted cells need to engraft at the site of injury, receive blood supply, and need to couple and communicate with cardiac cells of the host myocardium in order to ensure synchronous electrophysiological and contractile function. CMs derived from human iPSCs would even provide an autologous source of cells for future cardiac regenerative therapy[80]. However, heart repair by CM replacement appeared to be challenging and many hurdles need to be taken. These promises and challenges for hPSC-derived cellular therapies for the treatment of heart failures have been reviewed elsewhere[78, 79, 81]. One crucial aspect that we would like to emphasize here, is the composition of the transplanted cell population. Which cell type(s) or tissue constructs would be most ideal regarding cell survival, tissue repair, engraftment, and functional coupling? For the delivery of organized cardiac tissue we would require scalable cultures of different cell types, such as endothelial cells, smooth muscle cells, cardiac fibroblasts, and cardiomyocytes[82]. Another important aspect is the immature phenotype of hPSC-CMs. It is expected that a higher degree of maturation of transplanted cardiomyocytes and thus a higher resemblance to the native myocardium would pose a less arrhythmogenic risk[83]. For cellular therapy, these major basic research challenges are important to be overcome.

3.1 Fluorescent Reporters to Track Cells after Transplantation

In cellular transplantation studies, it is crucial to monitor cell delivery, homing, survival, proliferation, and migration of transplanted cells[84]. Another important aspect is electrical coupling of transplanted cells with host myocardium at single cell level. Impaired communication between transplanted and host cardiomyocytes may lead to electrophysiological disturbances and consequently cardiac arrhythmias. In order to study both homing and electrical coupling, the use of a dual reporter, combining bioluminescence imaging and a fluorescent reporter under the control of a cardiac promoter has been suggested[85]. However, most studies have used a single reporter line expressing either GFP or LacZ in hPSC-derived cells[80, 83, 86, 87]. Alternatively, direct cell labelling with fluorescent probes prior to transplantation, or local or systemic injections using non-invasive radionuclide imaging, magnetic labelling, or optical mapping through bioluminescence imaging are other options[85, 88]. Limitations for cell tracking that are important to consider are the half-life of the tracking label, dilution effects during cellular division and the take-up of the label by other cells after donor cell death. More details on the advantages and disadvantages of different cell tracking techniques are reviewed elsewhere[88].

3.2 Functional Integration

Functional integration of donor cells into host myocardium can be measured by the use of fluorescent calcium dyes, such as small molecule fluo-4[86]. Calcium is a vital component of cardiac excitation-contraction coupling. A wide range of small molecule calcium dyes is available, with different excitation/emission spectra and affinities for calcium. When choosing a calcium dye, the affinity for calcium should be taken into account, as low-affinity calcium dyes will provide more accurate measurements of calcium dynamics (reviewed elsewhere)[89]. Moreover, high affinity dyes may interfere with the physiological calcium handling, affecting function of transplanted cells, and thereby not completely reflecting the final engraftment conditions. Another option is the use of genetically encoded calcium indicator proteins, which allows cell-specific calcium mapping over a long time period[90, 91]. This approach was recently followed in two studies where hESC-CMs were transplanted in infarcted non-human primate hearts or guinea-pigs[83, 92]. Fluorescent calcium indicator GCaMP3 was incorporated in hESCs at the adeno-associated virus integration site 1 (AAVS1), a region of the genome that is considered to be transcriptionally constitutive active, using zinc-finger nuclease (ZFN)-based genome editing. Here, they demonstrated electrical coupling between donor cardiomyocytes and host myocardium. However, no significant increase in ejection fraction was found and hearts suffered from ventricular arrhythmias. The main advantages and drawbacks of the different fluorescent calcium-indicators described in this section are reviewed elsewhere[89].

3.3 Cardiomyocyte Proliferation

For a long period of time, the field of cardiac regeneration has been focussing on exogenous cell delivery and endogenous activation of cardiac cells to repair the damaged heart after infarction[78, 79]. Many studies have indicated that long-term beneficial improvement of cardiac function is an immense challenge.

Although several studies indicated a low proliferation rate of adult cardiomyocytes[93, 94], it is clear that mammalian adult cardiomyocytes have lost the capacity to regenerate the heart after damage, as opposed to fish, amphibians and fetal or neonatal mammalian cardiomyocytes[95, 96], therefore it is of importance to develop strategies that may lead to proliferation of cardiac progenitors (followed by differentiation to cardiovascular cells) and/or cardiomyocytes. Analysis of cell division could be performed through the use of live mitosis markers, such as scaffolding protein anillin, which is a component of the contractile ring that localizes to specific subcellular compartments during mitosis and cytokinesis. Hesse et al. fused anillin to fluorescent protein eGFP, to visualize high spatiotemporal resolution of mitotic phase, and proved a lack of cardiomyocyte division after myocardial infarction (MI) in mouse hearts[97]. Similarly, a fluorescent anillin reporter would be very

valuable for studying real-time cardiomyocyte cell division *in vitro* **(Figure 3)**. In addition, the fluorescence ubiquitination cell cycle indicator (FUCCI) is another system that enables live-imaging of cell division, based on a two-color (red and green) fluorescent proteins fused to cell cyle regulators ct1 and geminin, which are differentially expressed and ubiquinated dependent on the cell-cycle stage. This leads to a dynamic color change when cells are progressing from the G1 to S phase of the cell cycle. In contrast to an anillin reporter, the FUCCI system is not suitable for demonstrating cytokinesis[98].

4 The Promises and Challenges of hPSC-derived CMs for Disease Modeling, and Drug Discovery and Toxicity Screenings

Besides the opportunity to use human stem cell-derived cardiac cells for cell-based therapies, the utility of hPSC-CMs for disease modelling, drug discovery, and toxicity screenings has been reported in a growing number of studies, allowing researchers to study patient and/or disease-specific drug responses. hPSC-CMs are a promising alternative for animal models, as they recapitulate native human cardiomyocytes, indicated by expression of cardiac-specific genes and proteins, morphology and function. Further, they beat spontaneously, they possess calcium transients and they generate action potentials that are specific for atrial, ventricular, and nodal cells[99]. However, the use of hPSC-CMs as a platform for drug discovery need further optimization and validation and thus requires the development of tools to faithfully recapitulate cardiac physiology and function at high throughput level[100, 101]. One of the major significant challenges that scientists are facing is the mixed population of cardiac cells that are obtained from directed differentiation protocols
[100, 102-104]. Therefore, it will be important to generate specific differentiation protocols in order to obtain sufficient numbers of disease-relevant subtypes of cells, which is described in detail in a previous section. Moreover, for development of advanced 3D cardiac cultures with the purpose to increase the predictability of screening assays by mimicking the heart more accurately, it is important to understand the cardiac microenvironment, including cellular and non-cellular compositions and cell-cell interactions[100].

4.1 Cardiomyocyte Electrophysiology: Voltage and Calcium Dyes

Currently published hPSC-CM disease models, including channelopathies, cardiomyopathies, and metabolic cardiac disorders, show affected (drug-induced) action and field potentials, calcium signalling, disorganized sarcomeric structures, and metabolic abnormalities such as irregular lipid or glycogen levels[105-107]. Intracellular electrophysiological recordings of single cell hPSC-CMs by patch-clamp analysis provide detailed information about their action potential and the contribution of the ion channel currents. This has gained interest, since recent development of automated medium to high-throughput whole-cell patch-clamp chip recordings[108]. An alternative approach for this labour-intensive technology is extracellular non-invasive recordings using microelectrode arrays (MEAs), which is appropriate for long-term electrophysiological analysis, and is available for high-throughput screening. This will allow analysis of CM depolarization and repolarization from multicellular field potential recordings. In addition, the use of voltage-sensitive dyes represents a technology to monitor real-time electrophysiological activity at high temporal and spatial resolution[109]. These fluorescent reporters reside within the cell membrane and are able to monitor membrane potential, based on fluctuating fluorescent intensities. As these measurements can be performed at high throughput level, voltage-sensitive dyes-based assays are of high interest for drug screening in conditions that affect electrophysiological properties, such as channelopathies or drug-induced cardiotoxicity (see below). In addition, calcium handling is another important functional parameter of CM electrophysiology, for which fluorescent dyes and reporters are available. Calcium signalling in CMs is crucial for excitation-contraction coupling. During the action potential there is a calcium influx into the cell through activation of L-type voltage-gated Ca2+ channels. This results in calcium-induced calcium release from intracellular stores of the sarcoplasmic reticulum leading to contraction. Uptake of calcium from the sarcoplasm to the sarcoplasmic reticulum (in addition to other mechanisms that lead to reduced sarcoplasmic calcium levels) ends the contraction. Fluorescent calcium dyes, also described in a section above, include small molecules such as Fluo-4, Fluo-3, or Fura-2, or the more technical challenging Fura-4F, which has the advantage of a lower affinity for calcium binding, but requires switching between emission filters when calcium concentrations change[110]. Using small molecule calcium dyes, several studies have reported affected calcium handling in hPSC-CM disease models[107]. In the majority of disease modelling studies, single parameters are measured in a sequential manner. However, in order to thoroughly understand the deeper mechanisms behind the onset of disease and its phenotype outcome, it will be of interest to measure different crucial parameters simultaneously. The combination of fluorescent reporter/dyes with non-overlapping spectra, and technological advances in LED illumination and optical filters, will make this feasible for future applications.

4.2 Cardiac Toxicity

Drug-induced cardiotoxicity of both cardiac and non-cardiac drugs is a major problem in the process of drug development and may lead to unexpected life-threatening situations[111]. For the identification of cardiotoxic effects of drugs, it is important to understand the underlying mechanisms[112]. Since current animal models and cell lines lack sufficient predictability, hPSC-CMs may represent a valuable human-based *in vitro* model for preclinical cardiotoxicity screenings. Cardiomyocyte functional readouts may include calcium and voltage levels, sarcomere contraction forces and organization, ion channel functioning, CM apoptosis, mitochondrial damage, or other putative affected signalling pathways. Cellular mechanisms that have been associated with cardiotoxicity include genotoxicity, oxidative stress, apoptosis, and lipid peroxidization[113, 114]. Automated microscopy platforms in combination with fluorescent reporters/dyes enable quantification of a variety of cardiotoxic effects, such as nuclear fragmentation or caspase 3 activity to detect apoptosis,

mitochondrial membrane potentials[115-117], biochemical kinase assays based on FRET technology or specific signalling pathway analysis based on reporter assays fluorescent probes to measure cellular oxidative stress[118, 119], or fluorescent probes to measure superoxide, produced by mitrochondria under ischemic/oxidative stress conditions[120, 121]. Companies from whom such assays are available include Toxys, LifeTechnologies (Cellular Pathway Analysis Assays or CellROX), or Cyprotex. In recent years, the availability of disease-specific hPSC-CMs is growing, which allows new opportunities in the field of drug development and discovery. The demand for human-based standardized and validated cardiotoxicity assays is high and the development of reliable and sensitive pathway-specific-fluorescent reporter lines in well-characterized hPSCs in the combination with high content screening analysis methods, will be promising for medium- or high-throughput screenings of cardiotoxic side effects in the field of drug discovery[115, 122].

4.3 Optogenetics

A rapidly emerging field in biotechnology is optogenetics, which allows spatiotemporal activation of signalling cascades using green algae-derived light-sensitive cation channels, the so-called channelrhodopsins (ChR)[123]. Following activation with blue light these ChR allow the inward transfer of ions, leading to depolarization and local induction and propagation of action potentials in cardiomyocytes[124, 125]. This optogenetic approach can be followed to control electrical stimulation of heart muscle and to study subsequent cellular physiological responses under healthy and diseased conditions[123, 125, 126]. Moreover, by generating hPSC-derived channelrhodopsin-expressing nodal cardiomyocytes the realization of a light-activated bio-pacemaker may be feasible in the future. In addition, a recent innovative approach has been developed based on G-protein coupled receptors (GPCRs) containing motifs enabling light-induced spatiotemporal activation of GPCR-dependent pathways[127-129]. This will lead to a more in-depth understanding of specific intracellular signalling pathways in development, differentiation, and disease, and allows the identification of GPCRs and their downstream intracellular signalling cascades as therapeutic drugs target(s). Attributable to its ability to precisely control specific signalling pathways, optogenetics is a very promising technology for development of new assays in the field of drug development and toxicity, especially in combination with future 3D-disease modelling.

5 Cardiomyocyte Maturation and Sarcomeric Structures

One of the biggest challenges for the use of hPSC-CMs for preclinical drug screening is to increase the degree of maturity of hPSC-CMs[130]. Although morphological and functional aspects of hPSC-CMs resemble those of fetal cardiomyocytes, they still show a lack of maturity; hPSC-CMs are smaller in size, their sarcomeres, the smallest contractile units of striated muscles, have shown to be less organized when compared to adult CMs, they exhibit a lower contractile force and immature electrophysiological properties, and their metabolism is based on glycolysis, whereas adult CMs mainly use fatty acids as metabolic substrate[130, 131]. The lack of a mature phenotype may affect mechanistic and functional aspects of hPSC-CMs and consequently may result in a different drug-induced or disease-specific response. A higher degree of maturity is therefore most likely important for disease modeling and drug screening[100, 132]. Strategies to increase maturation in order to mimic human adult cardiomyocytes are followed with much interest. In addition, sarcomeric organization can be affected in hPSC-CM disease models and drug-induced cardiotoxicity[107]. It will therefore be of interest to study the organization of sarcomeric structures in hPSC-CMs with respect to the degree of maturation, and disease- and/or toxicity-induced sarcomeric disorganization **(Figure 4)**. In order to allow live-imaging for detailed analysis, a reporter line harbouring a fluorescent protein fused to sarcomeric proteins, may be a valuable additional tool, provided that the fusion protein will not affect cardiomyocyte function[133].

6 Conclusions and Future Outlook

Here, we reviewed the role of fluorescent hPSC reporter lines to our growing insights into cardiac differentiation, and their applications in the fields of cardiac regeneration, drug discovery, and toxicity screening. Fluorescent hPSC reporter lines have shown their advantages in the development and optimization of efficient cardiac differentiation protocols and will also be of high interest for deciphering the molecular signatures and mechanisms of differentiation of cardiac subtypes **(Table 2a)**, using technologies such as epigenetics (histone modifications, chromatin dynamics), transcriptomics, proteomics and metabolomics[134]. The use of dual- or multi-fluorescent reporter lines will have an additional advantage. In this manner, sequential lineage decisions can be visualized in real-time, which can be combined with in-depth molecular and functional single cell analysis[135].

A deeper understanding of the processes that determine maintenance and self-renewal of cardiac progenitors and thereby may provide an unlimited source of functional cardiac cells, will constitute an important contribution for the advancement of the fields of regenerative medicine, drug discovery and safety pharmacology. The identification of cell surface markers on specific cardiac cell subtypes allows enrichment of cardiomyocytes through cell sorting, which will be advantageous for the development of robust and validated assays for cardiomyocyte biology and/or function and for cell-based transplantation studies. In addition, improved knowledge on expansion and differentiation of cardiac progenitors may have far-reaching implications for the discovery of new therapies with a focus on activation of endogenous cardiac progenitors for the treatment of heart failure. In the context of heart repair it will also be of importance to visualize other biological processes such as cell death (necrosis and apoptosis) and the formation of cell-types following differentiation within their own lineage or as a result from switching cellular fate (e.g. via directed reprogramming). In this regard, subcellular labeling of cells using fluorescent proteins fused to specific localization signals, which target different organelles or compartments of the cell (e.g. membrane, cytosol or nucleus), offer an additional advantage for identification and

tracking of transplanted cells in complex multicolor labeling experiments.

We have discussed the advantages of fluorescent reporters in cell-based transplantation studies, indicated by the possibility to visualize engrafted cells (i.e. homing and survival) and to study functional integration and maturation into the host myocardium (Table 2b). Combination of these fluorescent reporter lines with other advanced reporter lines, such as those based on optogenetics, enables studying specific signalling pathways in a temporal spatial manner. In this context the use of the light-sensitive cation channels, channelrhodopsins (ChR), derived from green algae, allow the inward transfer of ions following activation with blue light, leading to depolarization of cardiomyocyte and local induction of action potentials that are propagated to electrically coupled neighbouring cells[124, 125]. This optogenetic approach can be followed to control electrical stimulation of heart muscle and to study subsequent cellular physiological responses under healthy and diseased conditions[123, 125, 126]. Moreover, by generating hPSC-derived channelrhodopsin-expressing nodal cardiomyocytes the realization of a light-activated bio-pacemaker is one step closer. Besides the *in vivo* applications, these reporter-based strategies have an important role for the further development and optimization of human *in vitro* assays for drug discovery, safety pharmacology and disease modelling.

Finally, use of reporter-based assays and cardiac cell-types facilitates the development of three-dimensional (3D) tissues or organ-like structures for tissue engineering and generation of highly advanced models that are more closely resembling the human heart using a multidisciplinary approach. It will be intriguing to see how, among others, the emerging and widely discussed 3D bioprinting technology may contribute to achieve this goal. In addition to cardiomyocyte subtypes, additional cardiac cell-types, including vascular cells and fibroblasts and the right composition of extracellular matrix components need to be included in order to generate these structures. Clearly, many hurdles need to be overcome, but increased interest of academia and industry in the use of human stem cell-based assays **(Table 2c)** will stimulate this field and likely increases the pace of its development, which will be a big step forward in improving efficient, cost-effective and safe drug development and effective therapies for the treatment of cardiac diseases.

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FIGURES

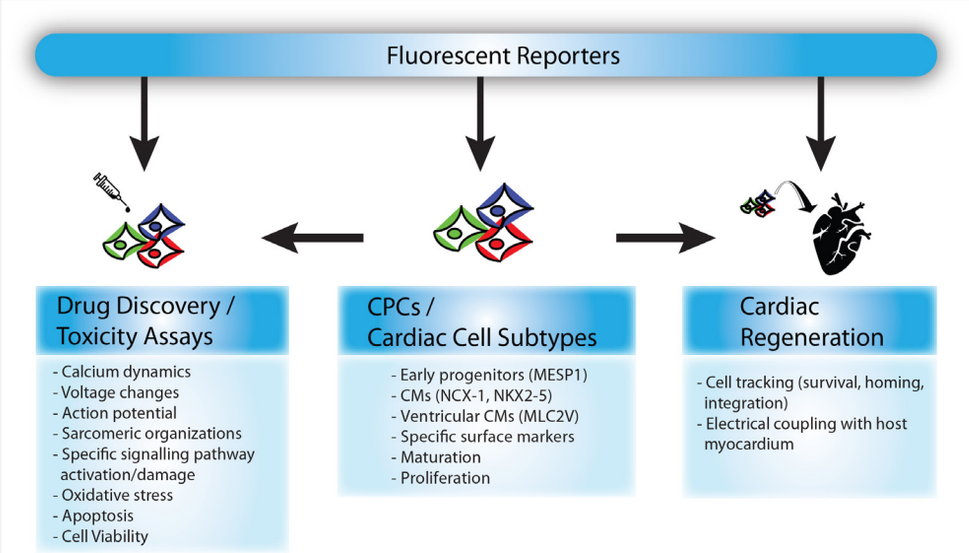

Figure 1. Schematic overview of Functional Applications of Human Fluorescent Reporter Lines for Cardiac differentiation and Cardiac Disease and Toxicity.

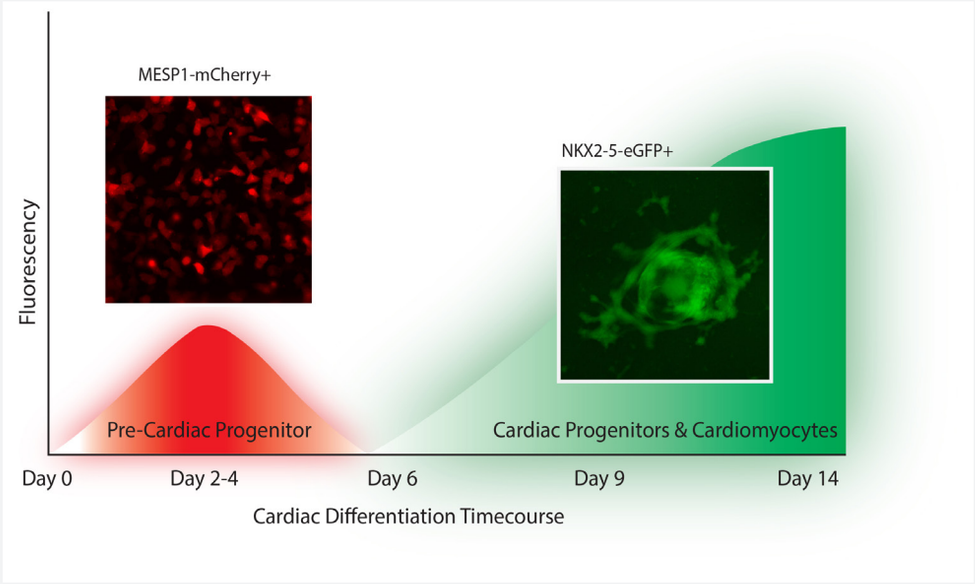

Figure 2. Dual Cardiac Fluorescent Reporter Line MESP1-mCherry-NKX2-5-eGFP in hESCs allows visualization and isolation of pre-cardiac mesoderm MESP1+ progenitor formation from hESCs, and their further differentiation to NKX2-5-eGFP+ derivatives, marking early cardiac progenitors and cardiomyocytes. *Den Hartogh et al. Stem Cells, 2015.*

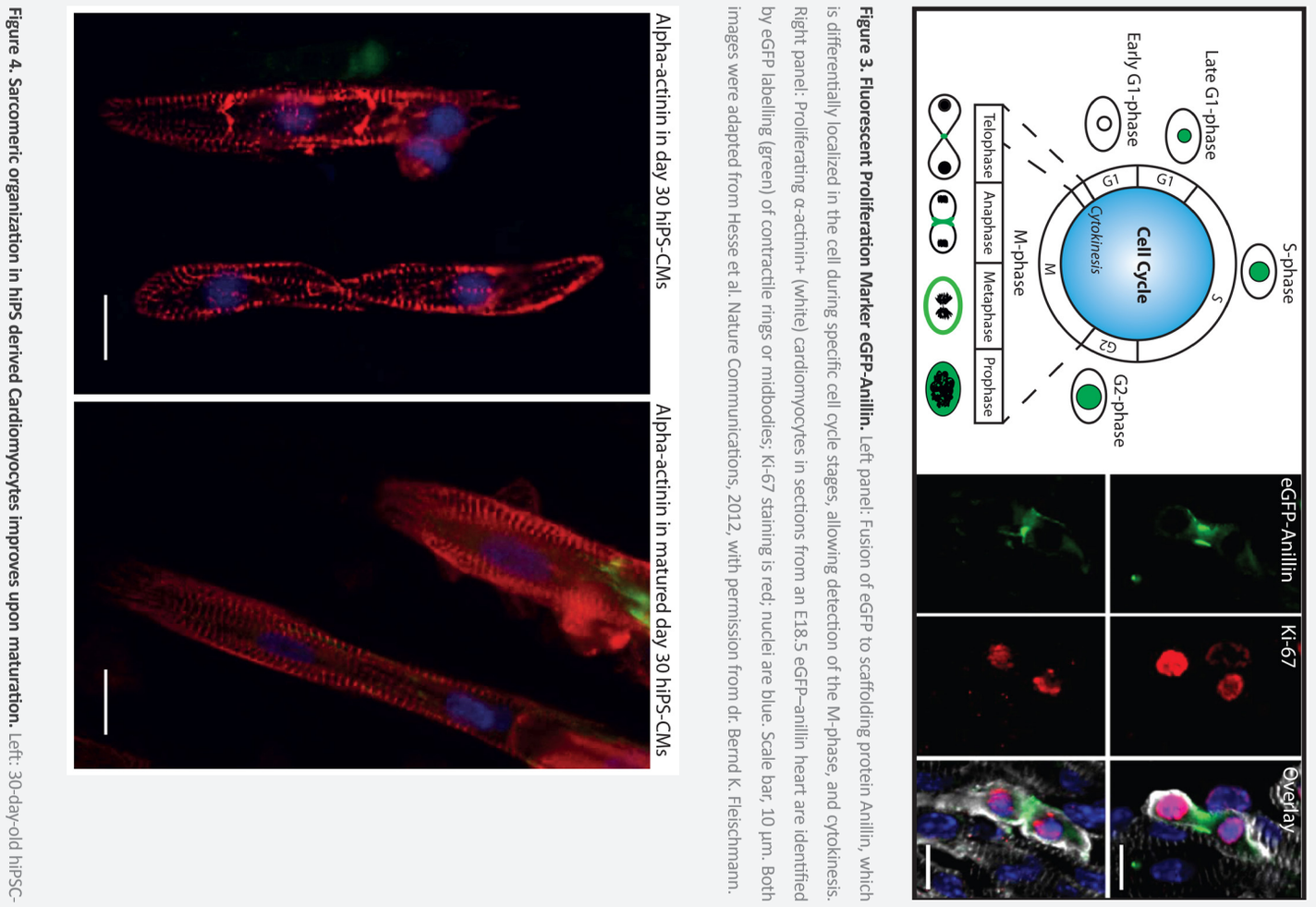

Figure 3. Fluorescent Proliferation Marker eGFP-Anillin. Left panel: Fusion of eGFP to scaffolding protein Anillin, which is differentially localized in the cell during specific cell cycle stages, allowing detection of the M-phase, and cytokinesis. Right panel: Proliferating α-actinin+ (white) cardiomyocytes in sections from an E18.5 eGFP–anillin heart are identified by eGFP labelling (green) of contractile rings or midbodies; Ki-67 staining is red; nuclei are blue. Scale bar, 10 μm. Both images were adapted from Hesse et al. Nature Communications, 2012, with permission from dr. Bernd K. Fleischmann.

Figure 4. Sarcomeric organization in hiPS derived Cardiomyocytes improves upon maturation. Left: 30-day-old hiPSC-derived CMs cultured in standard CM medium. Right: hiPSC-derived CMs after culture in commercial media optimized for cardiomyocyte maturation. Scale bar: 15uM. Ribeiro et al., Biomaterials, 2015.

	Targeting method	Key feature	Advantages	Disadvantages
Knockin/Phusion	Homologous recombination with DNA donor template	- Electroporation of target construct containing large homology arms around target region	- Endogenous gene reporter expression	- Efficiency depends on locus - Disruption of one allele of the gene
	Zinc Finger Nucleases (ZFNs)	- Pair of DNA binding proteins fused to endonuclease FokI - Donor plasmid	- Endogenous gene reporter expression - Efficient and precise	- Efficiency depends on locus - Disruption of one allele of the gene
	Transcription activator-like effector nucleases (TALENs)	- Pair of DNA-binding domains fused to cleavage domain - Donor plasmid	- Endogenous gene reporter expression - Efficient and precise	- Efficiency depends on locus - Disruption of one allele of the gene
	Clustered Regulatory Interspaced Short Palindromic Repeats System (CRISPR/CAS9)	- Cas9 endonucleases - Single guidance RNA (sgRNA)	- Requires only the generation of the sgRNA - Highly efficient in site-specific amino acid replacement	- Requires optimization for large elements - Risk for off-target modifications
Random integration	BAC	- Random integration of a large genomic sequence	- Large regulatory elements can be reported - Endogenous alleles stay intact	- Instable, risk for random integration of fragmented BAC elements
	Lentivirus	- Viral packaging particles containing reporter construct	- High efficiency of integration - Rapid	- Risk for silencing, thus analysis of multiple clones required - Position effects - Random integration may disrupt other gene functions - Only for in vitro applications - No control of copy number, site of integration, and gene expression level.
	Transposons	- Mobile genetic elements	- Non-viral gene delivery - Delivery of large elements	- Risk for hyperactive transposition - Risk for integration near transcriptional start sites
	Plasmid	- Electroporation or nucleofection of plasmid	- Non-viral gene delivery	- Only small regulatory regions can be reported - Position effects

Table 1. Advantages and disadvantages of the different approaches for generating fluorescent reporter lines.

	Gene	Regulatory elements	Fluorescent/Selection tag	Cell line	Method of integration	Plasmid/virus	Reference
Mesoderm	MESP1,NKX2-5	Endogenous	*Dual:* mCherry / eGFP	NKX2-5-eGFP HES-3	HR - knockin	Targeting construct	16
	Brachyury T	Promoter fragments	eGFP	hESC	Random	Lentiviral	6
CPCs	ISL1	Endogenous	*Tracing*: cre-IRES-puro / pCAG-flox-DsRed-MST	HUES3 / H9	HR - Knockin / Random	Targeting construct / Plasmid	44
	NKX2-5	Endogenous	eGFP	HES-3 and MEL-1	HR - knockin	Targeting construct	17
CMs	MLC2V	Promoter fragment	eGFP	hESC H9.2	Random	Lentiviral	64
	α myosin heavy chain (MHC)	Promoter fragment	eGFP-IRES-Puromycin, or eGFP-IRES-herpes simplex virus thymidine kinase/-ganciclovir (HSVtk/ GCV) suicide gene system	HUES-7	Random	Lentiviral	60
	α myosin heavy chain (MHC)	Promoter fragments	eGFP, mCherry, or Puromycin	hESC	Random	Lentiviral	6
	NCX-1	Promoter fragment	eGFP	iPS (C32 and C11 /hESC (H9 and Hes3)	Random	Lentiviral	59
Nodal-phenotype	Chicken Gata6	Promoter fragment	eGFP	H7 HESCS	Random	Lentiviral	71

Table 2a. Currently published human fluorescent reporter lines in the cardiac field.

		Advantage	Disadvantage
Cell Tracking/Homing	Consititutive LacZ expression	Good for studying homing	Not informative on electrical coupling with host myocardium
	Constitutive GFP expression	Good for studying homing and integration	Not informative on electrical coupling with host myocardium
	Direct cell labeling with fluorescent probes	No need for genetic modifications	Dilution upon cell division
	Non-invasive radionuclide imaging	Sensitive	Can not be used for long-term stem cell tracking
	Magnetic labelling	Excellent soft-tissue contrast	Low sensitivity, high cost of imaging, incompatible with the use of pacemakers (if used in clinic)
	Opical mapping - bioluminiscence	Good for studying homing	
Functional Integration	Calcium dye Fluo-4	- Electrical coupling with host myocardium - Fast response time	Limited tissue penetration
	Genetically encoded GCaMP3 calcium indicator	- Cell-specific calcium mapping - Ideal calcium indicator for *in vivo* models - Electrical coupling with host myocardium	Slow response time due to slow on-off kinetics

Table 2b. Fluorescent reporter lines for cellular therapy.

Assay	Measurement
Calcium dynamics	Intracellular calcium mobilization Calcium dyes: Fluo-2/3/4/8, Fura-2, Fura-4F (ratiometric)
Transmembrane potential	Voltage sensitive dyes
Cell viability assay	Hoechst expression / DNA binding fluorescent proteins
Apoptosis assay	Caspase3/7 activity through substrate luminescence
Oxidative stress	Reactive Oxygen Species (ROS)
Cell proliferation	Ki-67 eGFP-Anillin localizations PHH3 stainings
Hypertrophy / Sarcomeric organizations	Cell size Sarcomere staining
Intracellular lipid accumulation/ Peroxidization	Quantification of fluorescent intensity in cytoplasm (perioxidization assay kits available)
Signalling pathways	In vitro fluorescent reporter assays that reveil cellular response pathways

Table 2c. Fluorescent reporter lines for disease modeling, drug discovery, and toxicity.

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