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Review

Human stem cells as a model for cardiac differentiation and disease

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Abstract. Studies on identification, derivation and characterization of human stem cells in the last decade have led to high expectations in the field of regenerative medicine. Although it is clear that for successful stem cell-based therapy several obstacles have to be overcome, other opportunities lay ahead for the use of human stem cells. A more immediate application would be the development of human models for cell-type specific differentiation and disease *in vitro*. Cardiomyocytes can be generated from stem cells, which have been shown to follow similar molecular events of cardiac development *in vivo*. Furthermore, several monogenic cardiovascular diseases have been described, for which *in vitro* models in stem cells could be generated. Here, we will discuss the potential of human embryonic stem cells, cardiac stem cells and the recently described induced pluripotent stem cells as models for cardiac differentiation and disease.

Keywords. Stem cells, human embryonic stem cells, heart, cardiomyocyte, cardiomyopathies, channelopathies, differentiation.

Introduction

In recent years, there has been substantial progress regarding the identification, derivation and characterization of human stem or progenitor cells, comprising embryonic stem cells, adult and fetal stem cells and the recently described induced pluripotent stem cells (reprogrammed adult cells with stem cell-like properties). In general, stem cells have the capacity to self-renew and to differentiate to specialized cell-types. Advances in the stem cell field have led to high expectations with respect to their potential as a source for stem cell based therapies. Diseases or injuries caused by loss or damage of one or a few cell-types in organs that lack, or have limited capacity for self repair, such as neurodegenerative diseases, spinal cord injury, diabetes and heart diseases, have frequently been related to stem cell based therapies. Although significant progress has been made concerning directed differentiation protocols of stem cells and survival following transplantation, various hurdles have to be overcome before successful clinical applications can be realized [1]. In particular for the treatment of cardiovascular diseases, clinical trials have been performed using adult bone marrow stem cells, which resulted in mixed results, especially for long-term functional improvement [2]. For the treatment of heart failure in experimental animal models we and others have transplanted cardiomyocytes derived

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from human embryonic stem cells (hESC). Analogous to adult stem cell therapy, transplantation of hESC-derived cardiomyocytes (hESC-CM) led to short-term (four weeks) functional improvement [3–5], but was not sustained over the long-term (three months) [3], despite a three-fold higher number of transplanted hESC-CM [6].

Besides the potential of stem cells in the field of regenerative medicine, stem cells hold other promises. Stimulated by the progress in efficient, robust and reproducible differentiation protocols, genetic manipulation, and derivation of patient-derived stem cells, human stem cells provide a valuable tool for the study of early molecular events during directed differentiation or diseases. Since adult cardiomyocytes are terminally differentiated and primary cardiomyocyte cultures can not be maintained in culture, stem cell-derived cardiomyocytes offer a promising alternative for their use as an *in vitro* model system for cardiac development and disease. We will discuss different promising and clinically relevant human stem cells as a source for producing cardiomyocytes, which are: hESC, human induced pluripotent stem (hiPS) cells and human cardiac stem cells (hCSC). Other stem cells, such as mesenchymal stem cells, which have shown at least some degree of cardiac differentiation potential, are not considered in this review.

A stem cell-based cardiac differentiation model must satisfy several requirements to create a valuable and predictive model for cardiac development and disease. First of all, a robust and efficient cardiac differentiation model giving rise preferably to a homogeneous cardiomyocyte population is required. Secondly, to study molecular events in early cardiac differentiation and development, stem cell-derived cardiomyocytes should recapitulate signaling events during early embryonic cardiac development. Thirdly, stem cell-derived cardiomyocytes must function and respond in a similar manner as human endogenous cardiomyocytes. Fourthly, genetic manipulation of stem cells as a tool for following molecular events during differentiation or in response to different stimuli (e.g. using fluorescent reporter lines) is required. Finally, a high-throughput differentiation model under defined culture conditions will be necessary to efficiently screen small molecule or other libraries, in search of agents or drugs that affect the process of cardiac differentiation and/or disease (Fig. 1). Here, we will discuss the current state of the art concerning these issues and the steps that need to be taken in the future to create efficient and predictive cardiac *in vitro* models.

Stem cells as a model for cardiac differentiation and disease

Cardiac differentiation of hESC. Of all the different human stem or progenitor cell sources, hESC have been characterized most extensively. HESC were derived from donated blastocysts and characterized for the first time in 1998 [7]. Since then more than 400 hESC lines have been derived worldwide. HESC have the capacity to self-renew and therefore can be maintained as a cell line of undifferentiated cells. Furthermore, hESC are pluripotent, which implicates that they can differentiate to all cell types of the human body. However, it has become clear that hESC lines respond differently to directed differentiation protocols. In an international study (ISCI) 59 hESC lines were characterized and compared, demonstrating that all hESC lines had similar expression patterns for several hESC markers, despite their different genetic background and derivation techniques [8]. Nevertheless, variations of gene expression and imprinting status were observed for some genes. Whether these or other subtle differences affect their differentiation outcome and efficiency is not clear. Therefore, it is important to obtain robust and efficient differentiation protocols, which are applicable to multiple cell lines.

Since the first isolation of mouse embryonic stem cells (mESC) in 1981 [9, 10] much has been learned regarding their differentiation to cardiomyocytes. Although signaling pathways and culturing conditions in undifferentiated and differentiating embryonic stem cells are not identical between mice and humans, it was supportive for developing cardiac differentiation protocols in hESC lines. The classical approach for inducing cardiac differentiation from mESC is by formation of three-dimensional aggregates in a so-called "hanging drop" method (reviewed in [11]). These aggregates or embryoid bodies (EBs) contain differentiated cells derived from endoderm, mesoderm and ectoderm and can be further stimulated towards the cardiac lineage by combinatorial addition of several factors from the Wnt, BMP, TGFβ and FGF families (reviewed in [12]).

For the differentiation of hESC to cardiomyocytes two main protocols have been used successfully. One approach is based on classical mESC cardiac differentiation by EB formation, which has been further improved by serial application of activin A and BMP4 [5]. Alternatively, hESC are differentiated to cardiomyocytes by co-culture with a mouse endodermal cell line, END-2, resulting in the formation of beating areas within 12 days [13]. This differentiation system has been improved further by serum [14] and insulin [15] deprivation. In addition, differentiation in the

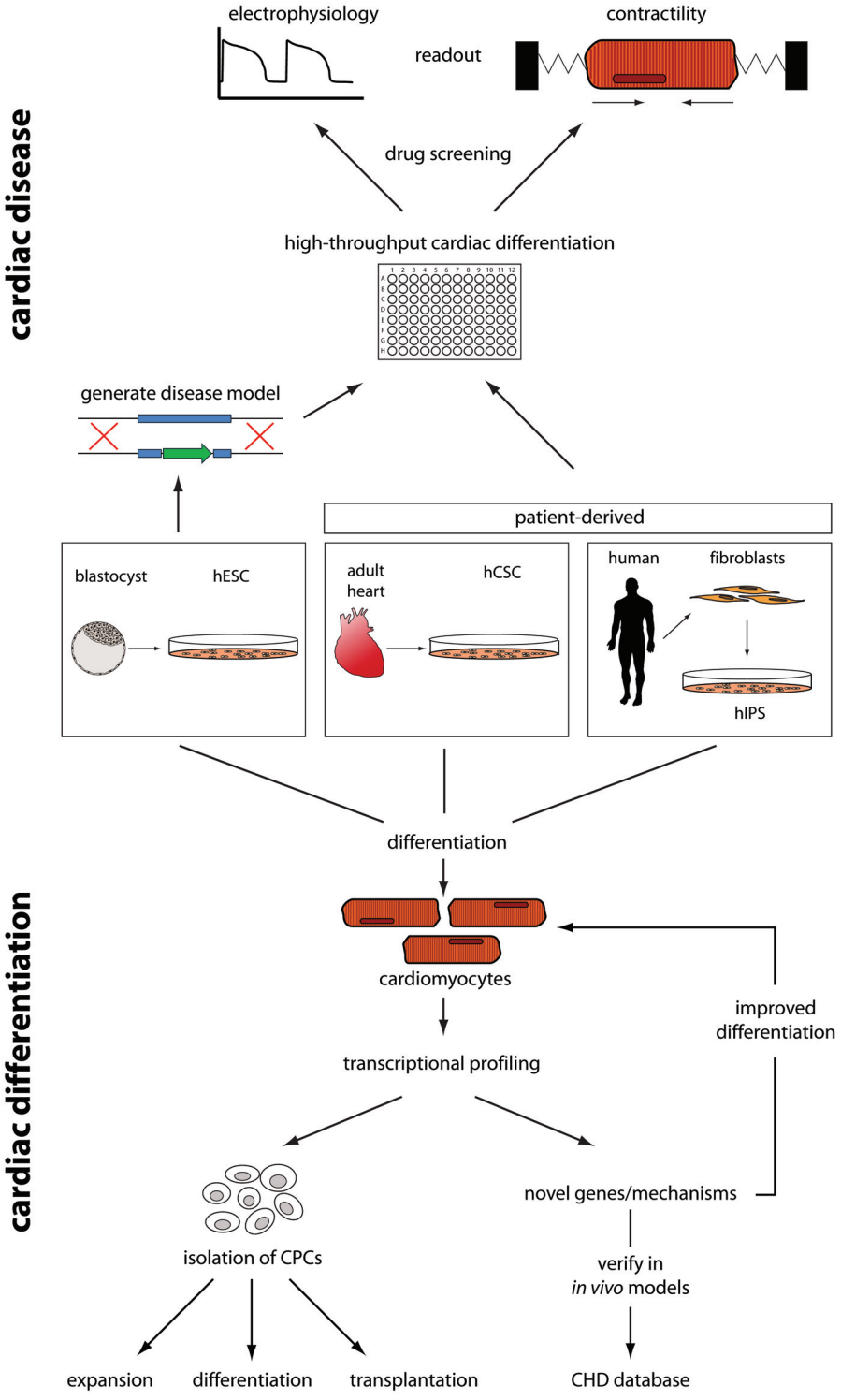

Figure 1. Human stem cells as a model for cardiac differentiation and disease. Schematic representation of the potential of different human stem cell sources. In the lower part of this figure stem cells are used as a model for cardiac differentiation. By transcriptional profiling, novel genes and mechanisms associated with different stages of cardiac differentiation may be identified and compared between the different stem cell sources, which may lead to: 1) the isolation of a subpopulation, such as cardiac progenitor cells (CPCs), 2) a better understanding of cardiac development by functional analysis in animal models and subsequently in databases of congenital heart diseases (CHD), 3) further improved cardiac differentiation *in vitro*. In the upper part of the figure, patient-derived stem cells are generated and subsequently differentiated in a high-throughput system to cardiomyocytes. Using readouts such as electrophysiology and contractility, the effect of drugs or small molecule libraries can be tested.

presence of END-2 conditioned medium has been shown to exert a similar cardiogenic effect on hESC, which could be further enhanced by inhibiting the P38 MAPK pathway [16]. In the same system, cardiogenic activity present in END-2 conditioned medium of END-2 cells could be mimicked in a chemically defined medium in the presence of prostaglandin I2 [17].

The manifestation of beating areas following differentiation of hESC suggests the presence of the

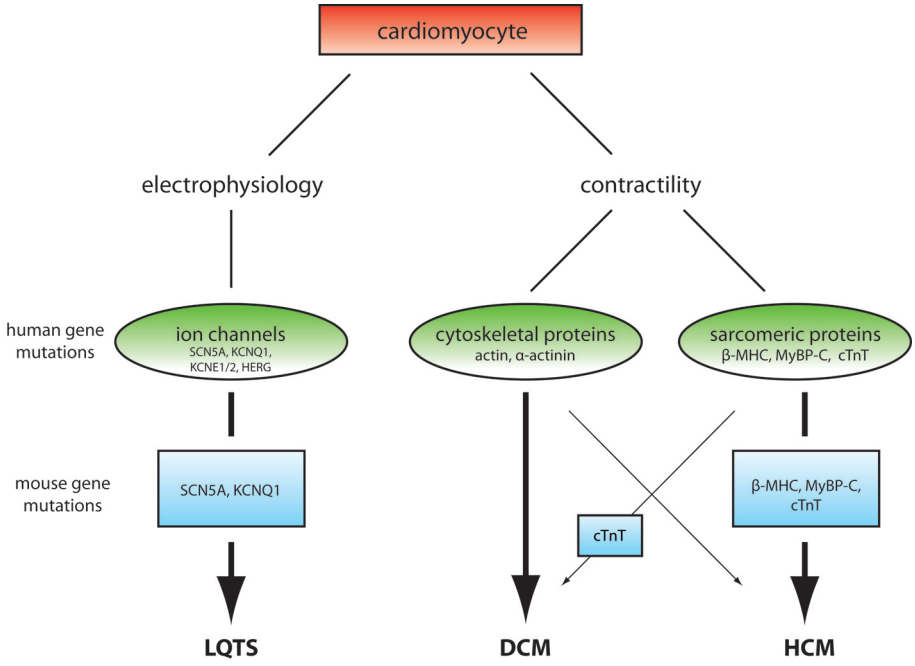

Figure 2. Mutations involved in cardiac channelopathies and cardiomyopathies. A diagram representing mutations affecting contractility and electrophysiology in cardiomyocytes leading to cardiomyopathies and channelopathies in humans (green) and their corresponding mouse models (blue).

appropriate ion channels and signaling and contractile proteins for functional excitation-contraction coupling. Several studies have confirmed this by molecular, biochemical, electrophysiological and pharmacological data. In general, hESC-CM display an immature phenotype resembling human fetal cardiomyocytes. HESC-CM respond to cardiovascular agents such as phenylephrine (α1-adrenergic receptor agonist), isoprenaline (β-adrenergic receptor agonist) and carbachol (muscarinic agonist). The main ion currents involved in the different phases of the action potential are Na^+, Ca^{2+} and K^+ (discussed below). The activity of these ion currents and expression of their respective ion channels have been demonstrated in hESC-CM (reviewed in [18]) .

Derivation and cardiac differentiation of hCSC. Previous studies in mouse hearts identified the presence of cardiac stem or progenitor cells, including so-called side population (SP) cells and cells that express the cell surface markers c-Kit and Sca-1 (reviewed in [19]). These findings prompted researchers to search for the presence of cardiac stem cells in humans. Messina *et al.* [20] described the isolation of a population of cells from atrial and ventricular biopsies, that formed a cluster of cells, called cardiospheres, which expressed several markers, including c-Kit. Differentiation to contracting cardiomyocytes was achieved by co-culture with rat neonatal cardiomyocytes. Recently, the efficiency of this isolation procedure was further improved [21]. Concomitantly, several other studies demonstrated the presence of c-Kit^+ stem cells from heart biopsies with the capacity to form cardiac cells *in vitro* and *in vivo* [22–24]. Recently, Goumans *et al.* [25] isolated a Sca-1^+ cell population from human fetal and adult heart biopsies using a mouse antibody. This Sca-1^+ stem cell population differentiated in three weeks to beating cardiomyocytes in the presence of demethylating agent 5-azacytidine. Adding TGFβ1 to these differentiating cultures further increased cardiac differentiation efficiency.

Generation and characterization of induced pluripotent stem cells. In 2006 Takahashi and Yamanaka [26] showed for the first time that forced retroviral overexpression of a cocktail of key stem cell proteins Oct4, Sox2, c-Myc and Klf-4 in mouse embryonic or adult fibroblast cultures led to reprogramming of these cell to pluripotent stem cell-like cells, called induced pluripotent stem (iPS) cells. Using an identical approach, the same group generated hiPS cells from adult skin cells [27]. Concurrently, Yu and colleagues achieved a similar result, by using transcription factors Lin28 and Nanog in addition to Oct4 and Sox2 in a lentiviral delivery system [28]. In these studies iPS cells exhibited essential characteristics of ESCs based on morphology, surface markers, gene expression profiles and telomerase activity. Further, iPS cell clones could be maintained in culture for at least several months and differentiated to cell types of all three germ layers *in vitro* and *in vivo* by the formation of teratomas in mice. Both mouse and human iPS formed cardiomyocytes. Since reactivation of c-Myc

increased tumorigenicity in mice, a protocol was developed for the generation of mouse and human iPS cells, without activation of c-myc [29]. One of the interesting prospects for the use of hiPS cells is cell-based treatment of patients with genetic diseases. This would involve repair of the defect in iPS cells, directed differentiation to the cell-type of interest, followed by cell transplantation. Since genetic material is derived form the patient, the risk of immune rejection is avoided (reviewed in [1]). The feasibility of this concept has been shown recently in mouse model of sickle cell anemia, a genetic blood disorder [30]. However, the more immediate applications of hiPS cells are likely to be development of human *in vitro* disease models for studying molecular mechanisms, drug screening and drug safety and toxicology.

Cardiac differentiation and transcriptional profiling. Studying the molecular pathways during directed cardiac differentiation of stem cells may, on the one hand, lead to the identification of novel genes that are important for cardiac differentiation, but on the other hand, may also reveal new candidates for congenital heart defects. Transcriptional profiling of differentiating stem cells is a powerful approach to study genes during various stages of directed differentiation. The first study to implement microarrays in cardiomyocyte differentiation was reported by Peng et al. [31] showing the global changes in gene expression in DMSO stimulated mouse P19CL6 cells. Although several cardiac-specific genes were upregulated during P19CL6 differentiation, the variability in cardiomyocyte differentiation of this mouse embryonal carcinoma cell line limits the predictive value of this model. Recently, mESC have been used to study cardiac gene expression by microarray analysis [32, 33]. In one study, mESC were differentiated into EBs with transient removal of serum which led to enhanced cardiomyogenesis. Microarray analysis showed that cardiac transcription factors, such as Nkx-2.5, Mef2c, and Gata4 were amongst the top 100 upregulated genes in differentiated EBs. Furthermore, 13 unknown genes were found highly expressed in EBs. However, this subset of genes did not show cardiac-specific expression by whole-mount *in situ* hybridization on mouse embryo's [32]. In another microarray study [33], purified cardiomyocytes, using a cardiac-specific α-myosin heavy chain (α-MHC) promoter driving EGFP expression, were compared to undifferentiated mESC. In total, 884 upregulated genes and 951 downregulated genes were found in mESC-derived cardiomyocytes. Unfortunately, no temporal gene expression was performed and novel genes were not further characterized.

Derivation of hESC lines and sequencing of the human genome made it feasible to study differentiation events in a human *in vitro* model using whole-genome approaches. Initial studies described the transcriptional profiling of spontaneously differentiating hESC to understand the genetic control of human embryonic development [34–36]. Most of these studies describe the comparison between undifferentiated hESC and differentiating EBs bodies, which provided more information about self-renewal of hESC than differentiation events. For example, Brandenberger et al. focused on pathways required for the maintenance of pluripotency and found that in particular FGF, WNT, NODAL and LIF pathways play important roles [34]. The first large-scale microarray analysis reporting temporal expression was performed on hESC and 2, 10 and 30-day old EBs [37]. By cluster analysis three clusters of genes were identified representing early, transient and late expressed genes. As is true for the previous studies, the lack of directed differentiation in this study prevents acquiring insights on transcriptional pathways that are important for embryonic development.

Until recently, the lack of efficient protocols for directed differentiation hampered large scale transcriptional profiling. As mentioned before, differentiation of hESC to cardiomyocytes is possible by EB formation or by co-culture with the visceral endoderm line, END-2. Improved cardiac differentiation efficiency and minimal variations in the timing and the number of beating areas in hESC-END-2 cultures made it possible to perform whole-genome microarray analysis. On average, beating areas contain approximately 25 % cardiomyocytes [13, 14]. Whole-genome microarray analysis was performed on different stages of cardiac differentiation on hESC-END-2 cultures and compared to a common reference pool. Furthermore, since hESC-CM highly resemble human fetal cardiomyocytes based on morphology, expression markers and electrophysiology [13], human fetal heart (hFH) was included as a reference source in this study, allowing to compare gene expression profiles of cardiomyocytes from differentiating hESC to that of hFH. By cluster analysis of the microarray data, genes were identified which were rapidly downregulated upon differentiation. Within this cluster of genes, known stem cell markers such as *OCT4* and *NANOG* were present, indicative of efficient differentiation. Furthermore, different clusters of gene expression were observed corresponding to early molecular events during embryogenesis, including the formation of mesoderm and endoderm, cardiac progenitors and fetal cardiomyocytes. In addition to identification of known cardiac transcription factors and sarcomeric genes, several novel genes, and genes not previously

associated with cardiomyocyte differentiation were identified and confirmed to be heart-enriched by means of whole-mount *in situ* hybridization in mouse embryos [38].

More recently, temporal expression of 468 genes that were previously reported to be expressed during cardiac development were compared in two different hESC differentiation protocols by microarray [39]. This led to the conclusion that EB based differentiation was more favourable for cardiac differentiation than an alternative, high density differentiation protocol. The same group provided a molecular signature of cardiomyocyte clusters derived from hESC by classifying genes according to their Gene Ontology annotation [40] and thereby showing high similarities to the molecular signature of human heart tissue.

In conclusion, transcriptional profiling of directed cardiomyogenic differentiation in hESC recapitulates *in vivo* temporal gene expression during cardiac development.

Identification of cardiac progenitors from differentiating stem cells. Sophisticated genetic methods such as Cre-Lox based lineage tracing and retrospective clonal analysis provided much information about the origin and fates of cardiac progenitors during embryonic development (reviewed in [41]). Cardiac progenitor cells express transcription factors Nkx-2.5 and Isl-1. By coupling these transcription factors to fluorescent proteins, investigators were able to isolate and culture these cells [42–44]. In these studies *in vitro* differentiated mESC were used for determining clonal origin. Differentiation to cardiomyocytes, vascular smooth muscle cells and endothelial cells showed that these isolated cells were true cardiac progenitor cells. Nkx-2.5$^+$ cells showed preference for differentiating in cardiomyocytes and smooth muscle cells [42], whereas Isl-1$^+$ cells gave rise to cardiomyocytes, smooth muscle cells and endothelial cells [43]. Recently, a similar Nkx-2.5-GFP reporter was used for isolation of cardiac progenitor cells in differentiating mESC. The clonally derived cardiac progenitor cells in their study expressed Nkx-2.5, Flk1 and c-Kit, but later also expressed Isl-1. These cells did show the capacity to differentiate to cardiomyocytes, vascular smooth muscle cells and endothelial cells [44], as opposed to the bipotent Nkx-2.5$^+$ cells from the previous study [42]. In that study the cardiac progenitor cells did not express Flk1, although a subpopulation of these cells expressed c-Kit. Another group used Flk1 as a marker for the isolation of cardiac progenitor cells. Following isolation, Brachyury$^+$/Flk1$^+$ cells from differentiating mESC were able to differentiate into the hematopoetic and cardiovascular lineage [45]. In an attempt to study cardiac progenitor cells in the postnatal heart, Laugwitz and co-workers identified Isl-1$^+$ cells in rat, mouse and human neonates, with the potential to form cardiomyocytes [46].

Recently, cardiac progenitor cells were derived from hESC [47]. To stimulate cardiac differentiation hESC were treated with combinations of activin A, BMP4, FGF2, VEGF and DKK1 in EBs. In analogy with their previous study in mESC, the investigators identified a KDR$^{+(low)}$ (Flk1)/ c-Kit$^-$ population, which showed cardiovascular differentiation *in vitro* and *in vivo*. These findings are in agreement with the previous studies on cardiac progenitor cells in mESC and with the identification of temporal gene expression patterns resembling the transition form mesoderm to cardiac progenitor cells [38].

At present, it is unknown whether the embryonic cardiac progenitor cells are the developmental precursors for the cardiac stem cells in adult hearts. At least a subpopulation of cardiac progenitors cells expresses markers that are present in cardiac stem cells, and vice versa. This remains to be studied in the future.

Genetic manipulation: creating tools for studying cardiac differentiation and disease. Unlike adult cardiomyocytes which do not proliferate and cannot be maintained in long-term culture, expansion of cardiac progenitors is likely the most efficient approach for generating higher number and purer cardiac cells. Isolation of progenitors can be achieved by using cell-surface markers for cell sorting (c-Kit, Sca-1, Flk1) or, if this marker is not available, by genetically marking these cells. In the latter approach, promoter or knock-in lines need to be generated coupled to a reporter gene, such as GFP, as already demonstrated for mESC. However, poor transfection efficiency in hESC has hampered progress in this area since the derivation of the first hESC line (reviewed in [48]). Several studies demonstrated high efficiency, using lentiviral infection [49], variable efficiency by plasmid transfection [50] and low efficiency, using adenoviral infection [51]. Additionally, homologous recombination, an important procedure for gene targeting, has also been achieved in hESC [52]. However, transfection efficiencies were very much dependent on which hESC line was used and since, at present, not all hESC lines are suitable for efficient differentiation to specialized cell-types, such as cardiomyocytes, it is crucial to develop generic, efficient transfection protocols. Costa and co-workers established an improved method for genetic modification by electroporation, the method of choice for gene targeting, in 4 different hESC lines [53]. Recently, improved transfection efficiencies have been achieved

in 12 independent hESC lines with different growth requirements. HESC lines were transiently transferred to feeder-free culture conditions allowing clonal growth and efficient gene transfer (80–90%) without loss of stem cell markers [54]. Stable lines retained normal karyotype and differentiation capacity. These technologies allow researchers to introduce reporter/selection constructs and generate genetic disease-causing mutations by homologous recombination into hESC.

Very recently, the first studies demonstrating the advantages of lineage-specific reporter hESC lines during differentiation, including cardiac, have been reported [55–58]. By lentiviral infection a cardiac-specific promoter (human myosin light chain-2V promoter) drove the expression of the reporter protein, eGFP. Differentiation to the cardiac lineage by EB formation followed by sorting of GFP^+ cells by FACS resulted in purer populations (> 90%) of cardiomyocytes [56]. In another study, a higher purity of cardiomyocytes was achieved by either negative selection (proliferation-associated suicide system, 33% purity) or by positive selection using the human α-myosin heavy chain (α-MHC) promoter coupled to a bicistronic reporter (GFP and puromycin, > 90% purity) [57]. Recently, a murine α-MHC promoter driving the neomycin-resistance gene was introduced into hESC followed by differentiation into cardiomyocytes and G418 selection, resulting in a high purity of cardiomyocytes [58].

Stem cells as a model for cardiac disease. The advantage of hCSCs and hiPS cells is that cells can be derived from the patient suffering from a genetic disease and therefore these stem cells do not need to be genetically modified. Moreover, the genetic disorder does not have to be known for generating an *in vitro* model and performing functional assays. However, it will not always be possible to obtain cell material, in particular for hCSC. And although hCSC can differentiate efficiently to cardiomyocytes, they can not be maintained as a cell line and therefore are dependent on new isolations, generating batch-to-batch-variations. Furthermore, in certain cases it would be advantageous to manipulate the genome, for example when a disease-associated promoter coupled to a fluorescent reporter is needed for a proper readout, or for rescuing the observed phenotype. It will be important to compare cardiomyocytes from patient-derived stem cells with cardiomyocytes from genetically modified hESC for the same genetic disorder.

The recently improved protocols for genetic modification in hESC will facilitate the generation of clinically relevant cardiac disease models. Successful homologous recombination has been shown for the first time by Zwaka and Thomson [52] and knocked out the HPRT gene on the X chromosome, leading to a complete loss of function in a female hESC line. Although it will be a matter of time, no cardiac disease models have yet been generated.

Towards a high throughput system. In order to generate disease models, it is important to optimize existing differentiating protocols and develop a defined high throughput model, suitable for screening small molecule or drug libraries. An initial attempt to create a robust and reproducible differentiation procedure was described by Ng et al. [59], using a known number of dissociated undifferentiated hESC in low-adherence 96-well plates, followed by centrifugation resulting in aggregation and the formation of EBs. In their study these so-called spin EBs or forced aggregates were transferred to culture plates for further differentiation towards the hematopoietic lineage. This differentiation procedure was adapted using a variety of feeder-free hESC lines, v-shaped 96-well plates and the addition of activin A and bFGF for differentiation towards cardiomyocytes [60]. However, a high interline variability in EB growth and cardiac differentiation was observed. Recently, a defined serum-free, animal product-free medium, denoted APEL (Albumin Polyvinylalcohol Essential Lipids) resulted in reproducible EB formation and differentiation with minimal batch-to-batch variation [61].

Models for cardiac diseases

Cardiac disease is one of the major causes of morbidity and mortality worldwide. With recent advances in the field of stem cell research, as discussed above, generation of human disease models are within reach. A prerequisite for predictive *in vitro* models for cardiac disease is the existence of a reproducible (preferably expandable) cell population that resembles the phenotypical characteristics of cardiac cells of patients in combination with consistent readouts, such as contractility and electrophysiology. In order to generate transgenic cell lines it would be advantageous to characterize single gene disorders. The most important characteristic of a cardiomyocyte is the conversion of an electrical signal to a mechanical response. It is therefore not surprising that many of the cardiac gene disorders are found in proteins that are important for force generation (cardiomyopathy) and electrical stimulation (channelopathy). The extrapolative value of *in vitro* models will be determined by comparisons to outcomes obtained from animal *in*

Table 1. Animal models of monogenic heart diseases with known clinically relevant mutations.

Disease model	Gene	Mutation	Species	Approach	Reference
HCM	α-MHC	R403Q/+	Mouse	Knock-in	[67–69, 92]
	β-MHC	R403Q	Rabbit	Transgenic	[93]
	MyBP-C	truncation	Mouse	Knock-in	[68, 71, 72, 94, 94, 94]
	cTnT	R92Q	Mouse	Transgenic	[74, 75, 79, 95, 96]
DCM	cTnT	ΔK210	Mouse	Knock-in	[77]
	cTnT	R141W	Mouse	Transgenic	[78, 79]
Duchenne	dystrophin	Premature stopcodon (base 3185)	Mouse	Spontaneous mutation	[97, 98]
LQTS/Brugada	SCN5A	1795InsD(h) /1798InsD(m)	Mouse	Knock-in	[82]
LQTS	SCN5A	ΔKPQ	Mouse	Knock-in	[81, 83, 84, 99]
	KCNQ1	A340E/+	Mouse	Knock-in	[88]
ARVD	Dsp	R2834H	Mouse	Transgenic	[100]

vivo models and ultimately from patients. Over the years many different animal models related to cardiac diseases have been generated. In particular, the mouse has been widely used as a genetic *in vivo* model. Although these models are important for our understanding of signaling pathways involved in the onset and progression of cardiac diseases, it is questionable how predictive these models are for drug discovery and development of new therapies. Human stem cell based disease models may provide a faster and cheaper model for initial drug screenings and may reduce the number of false-positive or false-negative results and may be complementary to the existing animal models. It is therefore important to compare future *in vitro* models for cardiac diseases with *in vivo* models. Next we will discuss current animal models for clinically relevant mutations or other genetic defects for the cardiovascular diseases, cardiomyopathies and channelopathies, which are related to impaired contractility and electrophysiology, respectively.

Cardiomyopathy. Cardiomyopathy is a disease of the heart muscle, with hypertrophic and dilated cardiomyopathy (HCM and DCM, respectively) as the most common forms. Features of HCM are left ventricular (LV) wall hypertrophy (increased size or volume of cells), enhanced systolic function accompanied with impaired diastolic function. On the histological level cardiomyocyte hypertrophy, myofibrillar disarray, and interstitial fibrosis is apparent. DCM is characterized by ventricular dilation, contractile dysfunction of the left and/or right ventricle(s) and the heart is hypocontractile due to impaired systolic and diastolic function. Histologically the major feature is myocyte disarray. About 25–30% of the DCM cases are familial and mostly inherited as an autosomal dominant trait [62, 63]. Genes that are mutated in HCM and DCM appear to be genes that encode proteins of the sarcomere (Fig. 2).

The sarcomere is the smallest functional unit responsible for muscle contraction, which is comprised of sliding thick and thin filaments. The thick filament consists of myosin and myosin binding proteins, whereas thin filaments are composed of actin, α-tropomyosin, troponins (C, I and T). The giant protein titin spans from the border (Z-line or Z-disc) to the midline of the sarcomere (M-line, comprised of thick filaments only) and provides, together with myomesins, scaffolding for the thick and thin filaments. Z-disc and cytoskeletal proteins are important for filament organization and assembly, but are also involved in transducing mechanical and chemical signals (reviewed in [64, 65]).

Mutations in genes that encode sarcomeric proteins have been found in patients with clinical features of both HCM and DCM. The majority of these mutations are present in proteins of the thin and thick filaments, such as beta-myosin heavy chain, cardiac myosin-binding protein C (thick filament) and cardiac troponins (thin filament), but are also described in titin and Z-disc proteins. In general, mutations in genes that encode proteins that are directly involved in force-generation are strongly associated with HCM, whereas mutations that affect proteins involved in force transmission from the sarcomere to the extrasarcomeric cytoskeleton are associated with DCM [62].

Animal models of HCM and DCM. For several mutations that have been described in patients with HCM or DCM, animal models have been generated (Table 1). Many mutations were found in β-MHC, the predominant isoform in human adult ventricles. The majority of these mutations are missense mutations, i.e. a single point mutation that leads to substitution of a different amino acid, and have been reported to cause severe forms of HCM. The most characterized mutation represents the Arg403Gln mutation. Since in smaller animals, including mice and rats, α-MHC is the predominant isoform [66] (α-MHC is abundantly

expressed in human ventricles and atria during embryogenesis), Geisterfer-Lowrance and colleagues generated a knock-in mouse model harboring the Arg403Gln mutation in the endogenous α-MHC gene [67]. Heterozygous α-MHC$^{403/+}$ mice displayed impaired cardiac function, evidenced by decreased cardiac output and delayed pressure relaxation and chamber filling [67–69]. By examining histological sections it was clear that the symptoms of HCM, such as myofiber disarray, hypertrophy and fibrosis, became gradually more severe with age in α-MHC$^{403/+}$ mice. In accordance, hypertrophic markers such as atrial natriuretic factor, brain natriuretic factor and α-skeletal actin showed increased expression [68].

Cardiac myosin-binding protein C (MyBP-C) is also frequently (25 %) mutated in HCM patients. Missense mutations as well as insertion/deletions leading to a C-terminal truncation of MyBP-C have been described [70]. Several knock-in mice models of the MyBP-C gene have been generated, including removal of exon 3–6 [71], exon 3–10 [72] and exon 30, which all resulted in truncated MyBP-C proteins. Homozygous mice for the exon 3–10 deletion displayed impaired cardiac function, cardiac hypertrophy, cardiomyocyte disarray, fibrosis and increased expression of hypertrophic markers. A similar phenotype was observed in mice lacking exon 30 of the MyBP-C gene, whereas a much milder phenotype was observed in mice lacking exons 3–6. In general, mouse models for truncated MyBP-C proteins showed much milder cardiac phenotypes, when compared to α-MHC$^{403/+}$ mice, resembling the severity of HCM in patients with mutations in MyBP-C and β-MHC genes.

Cardiac troponins, in particular troponin T and I, represent another group of proteins that have been associated with HCM and DCM. Missense mutations of cTnT in HCM patients show moderate cardiac hypertrophy, but have a poor prognosis and a high incidence of sudden death [73]. Transgenic mice expressing R92Q human TnT showed symptoms of human HCM, which were more severe with increasing transgene expression. The R92Q transgenic mice had significantly higher heart rate and diastolic dysfunction. Transgenic hearts showed cardiac fibrosis and cardiomyocyte disarray [74, 75].

In DCM patients, deletion of a lysine residue at position 210 of cTnT (ΔK210-cTnT) was reported to cause ventricular dilation and marked ventricular dysfunction, without signs of hypertrophic heart disease [76]. A knock-in mouse model of ΔK210-cTnT resulted in cardiac enlargement with marked ventricular dilation and systolic dysfunction in mice, which closely resembles the phenotype of DCM in humans [77]. In addition, two independent groups generated mice harboring a human R141W cTnT and showed clinical features of DCM [78, 79].

Cardiac channelopathies. The existence of an electrochemical gradient across the membrane of a cardiomyocyte makes it possible to initiate cardiac contraction by an electrical stimulus, the action potential, which is generated by the movement of ions across the membrane via ion channels. At the start of an action potential, or depolarization, the membrane becomes permeable for the movement of Na^+ ions from outside to inside the cell, resulting in an increase of the negative membrane potential. After this rapid depolarization phase, Na^+ channels close again, followed by a balance between inward Ca^{2+} and outward K^+ movements, resulting in a plateau phase. Subsequently, Ca^{2+} channels close, whereas more types of K^+ channels are opened, resulting in a negative membrane potential (repolarization phase), which finalizes the action potential. Genetic alterations leading to disturbed functions of cardiac ion channels are referred to as cardiac channelopathy (reviewed in [80]). Cardiac channelopathies can be either congenital (e.g. mutations in encoding genes) or acquired (e.g. induced by electrolytes, drugs, genetic predisposition). The congenital form can be caused by mutations in several different genes. Disturbances of the depolarization-repolarization events by ion channel mutations are more accentuated by prolongation of the action potential, which is characterized by a long QT (repolarization of ventricles) interval on an electrocardiogram. Patients with a long QT interval (long QT syndrome or LQTS) have an increased risk of arrhythmias, which may lead to sudden cardiac death. The majority of the known mutations in ion channels are associated with LQTS. In particular, genes encoding for potassium, sodium and calcium channels are most commonly affected (Fig. 2). Next, we will discuss the available animal models that mimic human congenital cardiac channelopathy.

Animal models of cardiac channelopathies. The SCN5A gene encodes the α-subunit of the cardiac Na^+ channel. Mutations in SCN5A cause LQTS by gain of function of the ion channel, leading to an increased Na+ current and action potential duration [81, 82] (Fig. 2). A deletion of nine base pairs of the SCN5A gene resulted in the deletion of three amino acids (KPQ), leading to LQTS. Accordingly, heterozygous mice lacking the same amino acids showed prolonged action potential duration and QT interval and early after depolarizations by ECG recordings [83, 84]. An insertion of the amino acid aspartate at position 1795 in SCN5A is associated with both LQTS and the Brugada syndrome, which is characterized by

an abnormal ECG and an increased risk of sudden death [85]. Remme *et al.* [82] generated a mouse model for this insertion. Heterozygous SCN5A 1798InsD mice resembled the clinical features of patients with a similar insertion, demonstrated by a prolonged QT interval although no cases of sudden death were reported. Another mouse model which shares similarity with the Brugada syndrome was generated by heterozygous deletion of the SCN5A gene ($SCN5A^{+/-}$). $SCN5A^{+/-}$ cardiomyocytes show reduced Na^+ current densities, slow conduction within the atria and atrioventricular conduction system, whereas the QT interval remained unchanged [86, 87]. Delivery of an extra stimulus induced ventricular tachycardia in $SCN5A^{+/-}$ mice and a response similar to pharmacological interventions [87].

Mutations in K^+ channels are frequently associated with LQTS. Many different mutations in KCNQ1, a gene that encodes the α-subunit domain of the voltage-gated K^+ channel, have been found and associated with LQTS [88]. Homozygous deletion of the KCNQ1 gene in mice results in inner ear defects and ECG abnormalities, including abnormal T-wave morphology, prolonged QT-interval and an increase in P-wave area and duration, which is comparable to the Jervell and Lange-Nielsen syndrome [89]. Casimiro *et al.* made different mouse models for missense mutations in the KCNQ1 gene. $KCNQ1^{T311I/T311I}$ mice had inner ear defects and only a mild cardiac phenotype with prolongation of QT intervals. $KCNQ1^{A340E/+}$ mice showed a prolonged QT-interval, which resembled the LQT1 syndrome in humans [88]. KCNE-/- mice displayed inner ear defects [90] and ventricular tachycardia, which could be induced by the addition of an extra stimulus [91]. Treatment with the L-type calcium channel blocker nifedipine suppressed ventricular arrhythmias in $KCNE^{-/-}$ mice and ECG showed that the electrogram duration was increased.

Concluding remarks

In this review we have discussed the possibilities of using human embryonic, induced pluripotent and cardiac stem cells as models for studying cardiac development and disease. Regarding cardiac development, in particular hESC but also cardiac stem cells provide a good model. Genomic profiling of differentiating stem cells demonstrated activation of gene clusters throughout the differentiation process, mimicking gene expression profiles during embryonic development in several species, related to gene expression profiles in epiblasts, gastrulation events and early cardiac differentiation. The information that is gathered for new genes that are associated with different stages of cardiac development can be further expanded by performing gain-and-loss of function in *in vitro* assays and *in vivo* experiments in different species (Fig. 1). The combination of gene expression patterns with functional data will provide further clues as to whether genes may be involved in congenital heart defects or other diseases. In addition, genes related to cardiac progenitors or cardiac mesoderm could be identified, indicated by the activation of transcription factors such as Mesp1, Nkx-2.5 and Isl-1. Promoters of these genes coupled to a fluorescent marker can be used to sort out a pure cardiac mesoderm or progenitor population. These purified cultures could be further used for directed differentiation to cardiomyocytes or used for cell-mediated therapy (Fig. 1).

Human ESC and iPS cells appear to be best suited for the generation of both disease models and associated medium/high throughput screening for identification of drug targets. However, a word of caution is warranted. At the present time hiPS cells derived cardiomyocytes are not fully characterized. Although they have been shown to form cardiomyocytes, there are still many unanswered questions. How efficiently do hiPS cells form cardiomyocytes? How do hiPS cell derived cardiomyocytes compare to hESC-CM and human cardiomyocytes regarding (epi-) genetic and protein markers and functional parameters, such as contractility and electrophysiology? How stable is their cardiac phenotype? Although hESC-CM are better characterized, to date we do not have sufficient information regarding their predictability as a model for cardiac disease and drug screenings. One disadvantage may be that hESC-CM resemble human fetal cardiomyocytes regarding molecular and functional parameters and therefore may behave differently to different stimuli than mature adult cardiomyocytes. To date, however, studies have reported responses of hESC-CM on different stimuli, which are comparable to these of adult cardiomyocytes. In future studies it will be important to compare cardiomyocytes from different cell sources in order to establish predictable *in vitro* models with high clinical relevance.

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