Understanding the Electrophysiological Maturation of Human Adult Cardiomyocytes

Lessons from the heart to be used for differentiation of pluripotent stem cells into cardiomyocytes

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The main focus of using human pluripotent stem cell derived cardiomyocytes (hPSC-CM) has long been on the field of regenerative medicine. The past decade the focus shifted towards using hPSC-CM as an *in vitro* model for screening of pro-arrhythmic compounds during drug development and to study cardiac arrhythmia-related diseases. The application of hPSC-CM as a new *in vitro* model requires a close resemblance between the physiology of hPSC-CM and human adult CM, especially regarding electrophysiology and contractility. We review the cardiac differentiation of hPSCs into CMs compared to the human fetal cardiac development. The comparison entails which transcription factors and signaling pathways are important in human cardiac development and applied in cardiac differentiation protocols. Moreover, electrophysiology and contractility of hPSC-CMs are compared with human adult CMs by evaluating gene expression and ion current recordings. An attempt is made to relate ion channel expression to transcription factors and signaling pathways expressed during human fetal cardiac development, to improve cardiac differentiation protocols producing more mature hPSC-CMs.

Key Words: cardiac differentiation, cardiomyocyte, contractility, electrophysiology, heart development, maturity, pluripotent stem cell

Introduction

The ability to derive human embryonic stem cells (hESC), with their capacity to differentiate into any cell-type of the three germ layers, became a very promising cell source for multiple purposes in a short time. The use of stem cells within the cardiovascular field, has long focused on regenerative medicine and its potential to treat cardiovascular diseases, like myocardial infarction (MI) and heart failure (HF) [for reviews, see [1, 2]]. However, preclinical animal studies and clinical trials have shown mixed results [3]. Possible reasons for these mixed results can be age, timing of the treatment and occurrence of the event (MI) [4-6]. Moreover, human pluripotent stem cell-derived cardiomyocytes (hPSC-CM), including both embryonic (hESC-CM) and induced pluripotent (hiPSC-CM) stem cell-derived cardiomyocytes, are a promising future *in vitro* model to study cardiac arrhythmia-related diseases and for screening of pro-arrhythmic compounds during drug development [7, 8]. Introducing human stem cells into routine drug development could reduce the necessity for animal testing, which is both ethically sensitive and costly. Moreover, it would circumvent problems with interspecies differences that impede the translation of the results to human [8]. To be useful as a screening model, hPSC-CM should resemble the human adult CM as closely as possible regarding contractility (contraction and Ca^{2+} cycling) and electrophysiology, including action potential (AP), ion channel expression, ion current density and ion current kinetics.

Current densities of ion current recordings of I_{Kr}, I_{Ks}, I_{Na} and $I_{Ca,l}$ in hESC-CM are comparable to adult CM. However, I_{K1} and I_f measured in hESC-CM do not resemble adult CM. I_f current density

is larger and a functional I_{K1} current is hardly present, explaining the membrane potential to be more depolarized in hESC-CM compared to adult CM [8, 9]. The same study by Sartiani et al. (2007) shows that the currents undergo developmental maturation in cardiomyocytes, which can be measured by modifications in current densities and properties of I_{to}, I_{K1}, $I_{Ca,L}$, I_f [9]. Another study reports similar findings regarding electrophysiological maturation over time [10]. Moreover, although contraction is measured, hPSC-CM have an immature phenotype regarding contractility compared to human adult CM. In particular, hPSC-CM have disorganized sarcomeric striations [11] and immature Ca^{2+} handling protein levels and Ca^{2+} cycling [12-16]. Overall, the phenotype of hPSC-CM is immature regarding electrophysiology and contractility. The immaturity of hPSC-CM might restrict the use of hPSC-CM in therapy, drug screening and as a model to study cardiac arrhythmia-related diseases.

The aim of this review is to compare the differentiation of hPSC into CM to the natural development of the heart, with focus on electrophysiology and contractility. In order to do so, a basic description of cardiac development is made, including important transcription factors, signaling pathways and gene expression during different stages of development. Thereafter, protocols used to differentiate hPSC into CM are compared to each other and to the known processes of normal cardiac development.

In vivo: Human heart development

The heart is one of the first organs to develop in an human embryo. While the heart first develops outside the embryonic disc, it will finally reside in the chest cavity due to folding of the embryo. Cardiac development is complex and many transcription factors and signaling pathways are involved, which will be discussed below. In Figure 1, an overview of transcription factors and signaling pathways are shown, both during human heart development (*in vivo*) and during cardiac differentiation of hPSCs into CMs (*in vitro*).

Early heart development

In human, the primordium of all three layers of the heart (epicardium, endocardium, myocardium,) first appear at the beginning of the third week after fertilization. The cardiogenic area is at the anterior part of the embryonic disk, where a collection of mesoderm cells reside (Carnegie stage 8, day 17-19). The mesoderm will give rise to the myoepicardial mantle, which differentiates into epicardium and myocardium. The mesoderm will be divided into somatic (outer layer) and splanchnic mesoderm (inner layer) (Carnegie stage 9, day 19-21). The myocardium will develop from the splanchnic mesoderm and is responsible for the early contractile ability of the embryonic heart. The epicardium is derived from the mesodermal lining. The somatic mesoderm will give rise to the body wall lining and dermis. Between the mesoderm and the underlying endoderm is a network of cells by which the endocardium is derived. The formation of the mesoderm and the regulation of myocardial progenitor cells is under tight control by several cardiac genes.

Genes involved in mesoderm formation, differentiation and specification: from mesodermal progenitor cell to cardiac mesoderm

The earliest cardiac genes involved in regulation of myocardial progenitor cells, are Mesp 1/2 and fibroblast growth factor 8 (FGF8), which are expressed in the gastrulating mesoderm. Thus, the mesodermal progenitor cells have not yet resided in the lateral plate mesoderm. In the mesoderm, these genes are down-regulated and again up-regulated in the endoderm and cardiomyogenic precursors and later in the cardiac outflow myocardium [17]. Studies including gene knockouts, chimeras and conditional knockouts concerning these genes show that both genes are necessary for mesoderm migration during

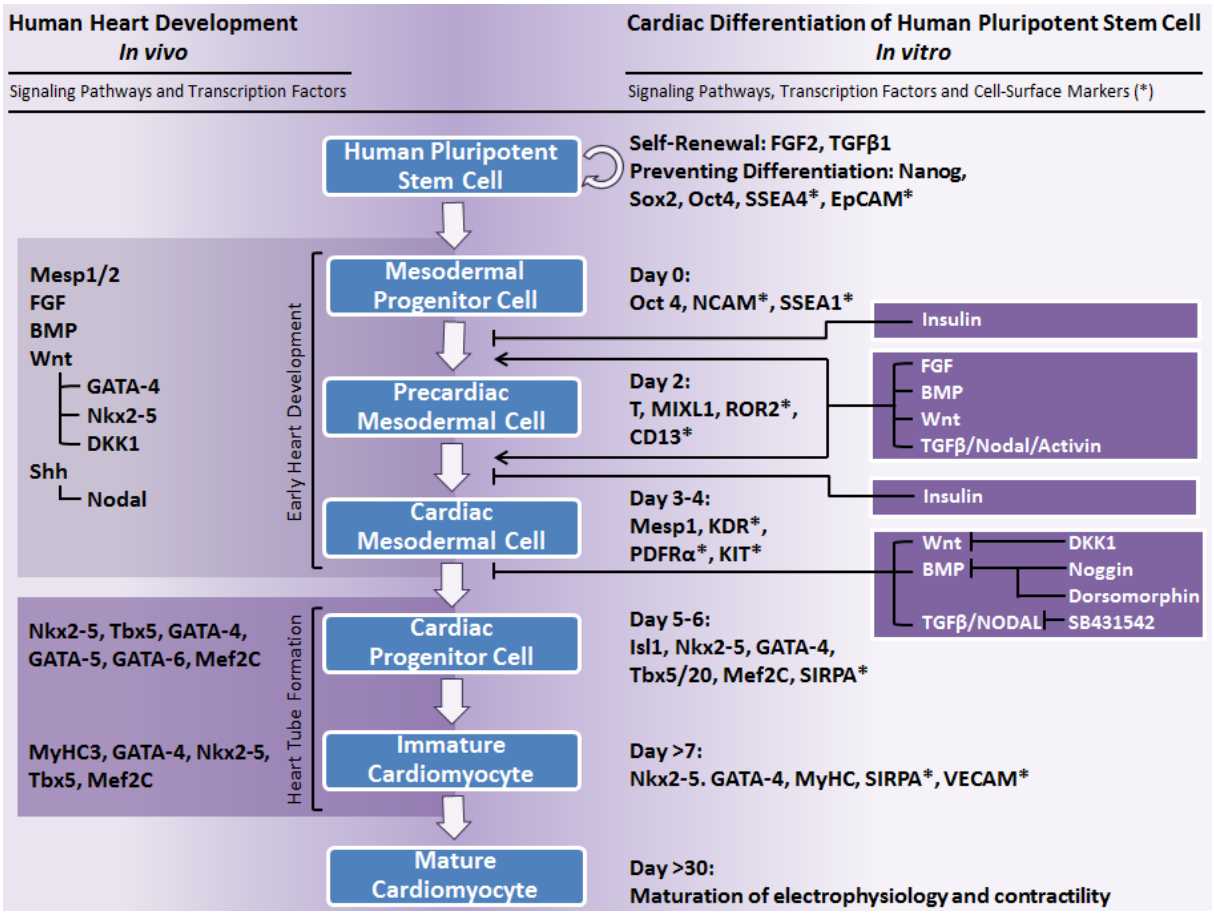

Figure 1. Cardiac differentiation of a human pluripotent stem cell (hPSC) via several progenitors into a mature cardiomyocyte (CM). The left side of the figure depicts human fetal cardiac development, including signaling pathways and transcription factors. The right side shows the major signaling pathways used during cardiac differentiation of hPSCs into CMs, including transcription factor and cell-surface markers (*). BMP, bone morphogenic protein; DKK1, Dickkopf 1; EpCAM, epithelial cell adhesion molecule; FGF, fibroblast growth factor; KDR, kinase insert domain receptor; Mef2C, myocyte enhance factor 2C; MyHC, slow myosin chain; NCAM, neuronal cell adhesion molecule; Nkx2-5, NK2 homeobox5; PDFRα, platelet-derived growth factor receptor α; Shh, sonic hedgehog; SIRPA, signal regulatory protein-α; SSEA, stage-specific embryonic antigen; T, Brachyury; Tbx, T-box transcription factor; TGFβ, transforming growth factor β; VECAM, vascular cell adhesion molecule.

gastrulation and that Mesp1/2 and FGF8 are important genes in heart formation and that down-regulation results in abnormal cardiac development [18-21].

Besides the signaling pathway involving FGF, bone morphogenetic protein (BMP) and Wnt proteins also play an important role in regulating the early stages of mesoderm formation and cardiogenesis. BMP promotes cardiogenesis by targeting the interaction between the zinc finger transcription factors GATA-4 and NK2 homeobox 5 (Nkx2-5) transcription factor via the mitogen-activated protein kinase (MAPK) Tak-1 [22]. Moreover, BMP can up regulate FGF8 and together may have a synergistic effect in cardiomyocyte differentiation from mesodermal cells [11]. Evidence shows that BMP is crucial to mesoderm and heart tube formation. When BMP signaling is antagonized by the protein Noggin, it causes a complete inhibition of differentiation of the precardiac mesoderm [23, 24]. Furthermore, heart tube formation is reduced or even absent after expression of dominant-negative BMP receptors in the mesoderm [25].

Non-canonical Wnt and sonic hedgehog (shh) signaling are believed to play a critical role in myocardial induction, while canonical Wnt signaling has an inhibitory effect on myocardial

facilitation [17]. Shh plays a critical role in the left-right asymmetry during heart tube looping. The first left-right axis of the heart is found during gastrulation. In the primitive streak, shh is expressed in the left side of Henson's node. However in the right side, expression is inhibited by the activation of Activin IIa receptors. When the mesoderm is formed, shh triggers expression in the left side of Nodal and Pitx2. During further development of the heart, Pitx2a is expressed on the left side of the heart tube and is responsible for asymmetric organogenesis. [26]

Non-canonical Wnt ligands, like Wnt11 which are expressed in the endoderm and mesoderm, stimulate cJun-N-terminal kinase (JNK) and thereby enhance myocardial differentiation. Furthermore, Wnt11 also inhibits canonical Wnt signaling. Wnt3A and Wnt8 are canonical Wnt ligands that activate β-catenin and thereby initiate canonical Wnt signaling repressing myocardial differentiation. During gastrulation. the node produces Dickkopf 1 (DKK1) and Crescent, which maintain a zone of low canonical Wnt signaling and thereby promote cardiogenesis[17].

Genes involved in cardiogenic specification: from cardiac mesoderm to cardiac progenitor cells

Nkx2-5 is one of the earliest markers expressed by precardiac cells which originate from the lateral plate mesoderm [22]. Nkx2-5 is involved in spatio-temporal expression of cardiac genes, not only during embryonic heart development but also during postnatal heart development [22]. It can interact with many other cardiac markers and has combinatorial activity. Nkx2-5 and T-box transcription factor (Tbx5) have a combinatorial action causing atrial cell fate specification. Moreover, Nkx2-5 can interact with zinc finger transcription factors of the GATA family (GATA-4 and GATA-5). GATA-4 is another important marker involved in spatio-temporal expression of cardiac genes and is also an early marker of the precardiac cell that is traceable 7 days after fertilization in the splanchnic mesoderm [27-29].

Furthermore, GATA-4 is expressed in the extra-embryonic endoderm and myocardium during heart tube formation and folding [30-32], and in cardiomyocytes throughout development and adult life [30]. Studies focusing on ablating GATA-4 in mice cardiomyocytes resulted in myocardial hypoplasia and defective heart tube formation [30, 33, 34]. Another study on GATA-4$^{-/-}$ ES cells showed a defect in its visceral endoderm, without any other defects in other aspects of differentiation [35, 36].

Several studies showed that GATA-4 is not necessary for initiation of cardiac differentiation, but showed that it is a potentiator for cardiac differentiation [35-37]. In case of GATA-4 ablation, there is increasing evidence that GATA-5 and GATA-6 take over the role of GATA-4. Moreover, the interaction of GATA-4 and Nkx2-5 can synergistically activate the atrial natriuretic factor (ANF) promoter, cardiac α-actin and cardiac-restricted ankyrin repeat protein (CARP) [22]. Furthermore, this interaction also plays an important role in postnatal development by maintaining cardiac gene expression and phenotype [reviewed by [22]].

GATA-5 is initially expressed in the precardiac mesoderm, where it plays a role in spatio-temporal cardiac gene expression. In later stages (stage 9.5) it is expressed in both atrial and ventricular endocardium and in stage 12.5 it is expressed only in the atrial endocardium. In late fetal development GATA-5 is no longer expressed within the heart [38].

In mouse, GATA-6 can only be found in the precardiac mesoderm and later in the heart tube [36, 39, 40]. GATA-6 in Xenopus can be detected already in the gastrulation mesoderm and later on in the precardiac cells. Overexpression of GATA-6 in Xenopus cardiac cells results in arrest of cardiac differentiation, showing its potential in maintaining the precursor status [41]. Notably, mouse embryos lacking GATA-4 and GATA-6 do not develop heart tissue [42].

Moreover, myocyte enhance factor 2 (Mef2), which plays an important role in CM differentiation by regulation of cardiac muscle structural genes, interacts with GATA-4 or GATA-6 and thereby activates downstream targets of which the ANF promoter [11, 22].

Studies in *Xenopus* embryos have shown that the factors Nkx2-5, GATA-4, GATA-5 and GATA-6 need to cooperate with the other factors in precardiac cells to be able to induce cardio-genesis, because these factors alone cannot induce cardiogenesis [43, 44].

Heart tube formation

The network consists of two branches (crescent shape) located bilaterally of the trilaminar embryo in the cranial region and as the body of the embryo folds, the branches meet and fuse forming a single heart tube at the end of the third week postcoïtum (Carnegie stage 10, day 21-23). The single heart tube has the shape of an inverted Y and from cranial to caudal position, the heart tube is segmented into the truncus arteriosus, bulbus cordis, primordial ventricle (stem of the Y), primordial atrium (arms of the Y) and sinus venosus. At day 22-23 the myocardium is contracting, pushing blood cranially from the sinus venosus. What used to be described as the second crescent within the embryonic disk, is now referred to as the secondary heart field, which populates the cells forming the outflow tract and the right ventricle [45]. Before the heart tube is formed the precardiac cells already express some cardiac specific genes, like genes which encode for contractile proteins. [22]

Once the heart tube is segmented into atrial and ventricular domains, transcription factors are expressed causing further differentiation of the myocardial progenitor cells into cardiomyocytes and continuing the development of the mature heart [17].

Genes involved in cardiomyocyte differentiation: from myocardial progenitor cells to immature cardiomyocytes

When the heart tube is formed, several factor are expressed exclusively in the atria or in the ventricles, like the expression of the slow myosin chain type 3 (MyHC3), which is involved in chamber specification. A GATA binding site is necessary for the MyHC3 promoter to be expressed in the atria, whereas a vitamin D response element (VDRE) is essential for inhibition of ventricular expression [46]

$Mesp^{+}$ cell subpopulations express the homeodomain transcription factor Nkx2-5, T-box protein Tbx5, LIM homeodomain transcription factor and Mef2C [11, 17]. These cardiac transcription factors are necessary for interpretation of the patterning signals within the primitive heart tube. Mice deficient in Mef2C show hypoplasia of both ventricles [47]. However, it needs to be determined whether this is also the underlying cause for ventricular hypoplasia in humans.

During mouse heart development, the related basic helix-loop-helix (bHLH) transcription factors eHAND/HAND1 and dHAND/HAND2 are mostly expressed in the primitive left and right ventricular segments, respectively [48, 49]. eHAND mutant mice did not give clear insights to its role in the heart. However, a deletion of dHAND in mice, caused hypoplasia of the primitive right ventricle [50, 51].

Moreover, it is suggested that eHAND acts downstream of Nkx2-5 to control left ventricular development. Mice deficient in Nkx2-5 failed to express eHAND in the heart and showed lethal defects in ventricular morphogenesis [52].

Finally, the immature cardiomyocyte will mature with time and development of the embryo. Maturation will include a more mature electrophysiology and Ca^{2+} handling, myofibril organization and sarcomeric striations [53].

Human fetal electrophysiology and contractility

Electrophysiology and contractility are key aspects for the heart to function. Due to the scarceness of human fetal cardiomyocytes, studies are often performed in animal fetal myocytes.

Electrophysiology

During early cardiac development, Na channels, giving rise to the fast sodium current (I_{Na}), are expressed in atrial and ventricular fetal murine myocytes. The biophysical properties of the Na^+ channels in these myocytes are similar to those in neonatal and adult murine hearts. Expression of the Na^+ channel is similar in ventricular and atrial cells at both early and late stages. However, I_{Na} maximal current density increases from about 10 pA/pF at -20mV in early stage (days 11-13) fetal murine ventricular myocytes to 115 pA/pF at -20mV in late stage (days 17-19) cells [54]. Similar to Na^+ channel density in fetal murine myocytes, channel density also increases significantly in rat and chick hearts [55, 56]. In another study, cell capacitance increased with 36% from day 13 to 19 with a 4.5 times larger increase in I_{Na} current density compared to L-type Ca^{2+} ($I_{Ca,L}$) current density within the same period [54].

L-type Ca^{2+} channel activity, responsible for $I_{Ca,L}$, is identified both in atrial and ventricular cells of early-stage embryonic mouse hearts and have similar current kinetics. The maximal current density measured in early stage (days 11-13 postcoitum) fetal murine ventricular cells is recorded at ±20 mV and is about 8,5 pA/pF. In late stage (17-19 days postcoitum) fetal murine ventricular cells have a maximal current density of 22 pA/pF at 20 mV [54]. Thus, L-type channel current density increases during days 11 through 20 of murine embryonic hearts. No T-type Ca^{2+} channel activity can be measured in atrial or ventricular fetal murine cardiomyocytes [54], which is contradictory to T-type channel expression in neonatal murine heart [57]. In rabbit ventricular myocytes, the maximal peak current density of $I_{Ca,L}$ increases significantly from day 21 of gestation compared to the neonate. However, the most significant increase in maximal peak current density of $I_{Ca,L}$ with a 3-fold increase, occurs from the neonate to the adult [58]. Furthermore, an increase of Ca^{2+} channel density can be found in fetal rat cardiomyocytes [59], whereas in fetal chick cardiomyocytes a decrease in channel density is observed [60]. In human, mRNA levels for the α1c subunit of L-type and α1h subunit of T-type Ca^{2+} channels are determined and show an increase in mRNA for the α1c subunit during development with a maximum at the adult stage, whereas the mRNA levels of the α1h subunit decreases and reaches the lowest levels in the adult stage [61].

In early stage ventricular fetal murine myocytes, only 75% of the measured cells have both Na^+ and Ca^{2+} channel activity. In 20% of the cells only Ca^{2+} channel activity is found and in one cell only Na^+ channel activity is measured. In the late stage fetal ventricular myocytes, all measured cells display both Na^+ and Ca^{2+} channel activity. The very low current density of I_{Na} and the fact that almost all cells display Ca^{2+} channel activity in the early stage of ventricular murine myocytes, suggests that L-type Ca^{2+} channels are the most predominant expressed channel concerning excitation at very early stages (< 11 days) of the fetal murine heart [54].

In early stage (day 11-13) murine embryonic myocytes, rapidly activating K^+ currents are measured in response to different test pulses with three distinct inactivation kinetics. The rapidly activating and inactivating K^+ channel is expressed in 50% of the early stage and in all late stage atrial fetal murine myocytes and in only 18% and 20% of the ventricular fetal murine cells of the early stage and late stage, respectively. The rapidly inactivating current is most similar to the transient outward current; I_{to}. The rapid activating, but slow inactivating K^+ channels are expressed to a much lesser extend in the atrial cells (25% in early stage cells and 1% in late

stage cells) compared to ventricular cells (54% in early stage and 45% late stage cells) [54]. Biophysical properties of the rapid activating and slow inactivating K^+ current resembled *Shaker*-type and *Shab*-type channels. Moreover, slow inactivating K^+ currents are similar to adult rat atrial delayed rectifier and neonatal canine epicardial delayed rectifier [54, 62, 63]. The percentage of cells expressing the very rapidly activating but non-inactivating K^+ channel and which may represent the component of native I_{to} insensitive to 4-AP, is about the same in both ventricular and atrial cells in early stage fetal murine mycotyes (±30%), hardly present in atrial late stage myocytes and in about 30% of the late stage ventricular myocytes. Complete pharmacological dissection of these rapidly activating K^+ currents was not performed in this study, making identification of these K^+ current difficult [54].

One study reports that hardly any cells are found expressing the slow delayed rectifier K^+ channel in early stage atrial and ventricular fetal murine myocytes. However, in the late stage cells 50% of ventricular myocytes had I_{Ks} channel activity.[54]

The most predominantly expressed K^+ channel, is the delayed rectifier. It is a voltage and time dependent current and the channel predominantly is expressed in the atria in the early stage fetal murine cells. In late stage cells, expression is about the same in atrial and ventricular fetal murine myocytes. No increase in expression levels is found during progression of development. The maximal current density of the pulse current is 1,1 pA/pF at 10 mV in atrial fetal murine myocytes. Adding the specific blocker E-4031 results in a complete reduction of the total delayed rectifier current in atrial fetal murine myocytes, supporting I_{Kr} is the sole delayed rectifier channel present in these atrial cells[54]. The biophysical properties of I_{Kr} in fetal murine myocytes resembles I_{Kr} in adult guinea pig cells [64], neonatal cardiomyocytes [57] and human atrial cardiomyocytes [65].[54]

The inward rectifier K^+ current (I_{K1}) is measured in 18% and 36% of early stage atrial and ventricular embryonic murine cells, respectively. Inward rectifying K^+ channel activity can be measured in all late stage cells, both atrial and ventricular. Similar to I_{Kr}, current density of I_{K1} does not increase during development. However, I_{K1} gene expression increases four-fold [54]. Similar developmental changes can be found in other species, like rats, rabbits and chick hearts [58, 66, 67].

Contractility

As discussed above, around day 22 to 23 after fertilization, the human heart tube starts to contract and thereby propelling blood cranially. The heart is not yet fully developed and is not able to sufficiently pump blood at this stage. Around day 28 after fertilization, the heart starts to beat and around day 33 the heart begins to descent towards the position in the thoracic region, similar to that in adults. Besides the immature contractility during early heart development, differences in functioning of Ca^{2+}-handling proteins are also reported.

Calcium handling

One report shows that Na^+-Ca^{2+} exchanger (NCX) mRNA and protein levels are higher in human fetal hearts than in adult hearts [68]. Sarcoplasmatic/endoplasmatic reticulum Ca^{2+} ATPase 2a (SERCA2a) mRNA levels do not significantly change from fetal to the adult stage of human hearts [61]. The same study did perform a western blot experiment using fetal (8-, 10-, 12-, 15-, 18- and 20-week gestation), neonatal (2 days after birth) and adult (40-year-old) hearts, showing increased levels of SERCA2a protein levels from 1 at 8-wk gestation to 2.5 at neonate and 3.2 at the adult stage [61], suggesting a difference in posttranslational processing.

Less L-type Ca^{2+} channel α1c subunit mRNA levels and more NCX mRNA and protein levels in fetal compared to adult CMs, supports the idea that in fetal and neonatal CMs sarcolemmal Ca^{2+} handling proteins may play an important role in

sarcolemmal Ca^{2+} entry [61]. On the other hand, the high T-type Ca^{2+} channel expression in fetal CMs compared to adult, might compensate for the low L-Type Ca^{2+} channel expression and thereby provides an additional route of Ca^{2+} entry through the sarcolemma [61].

In vitro: human pluripotent stem cells derived cardiomyocytes

hPSC-CMs resemble human cardiomyocytes in many aspects. However, still the hPSC-CMs exhibit an immature electrophysiological phenotype concerning several ion currents. Moreover, hPSC-CMs are also immature regarding several aspects of contractility. Giving an overview of which protocols are used for cardiac differentiation and with which electrophysiological outcome, might provide new insights in improving cardiac differentiation of hPSCs.

Cardiac differentiation methods for human pluripotent stem cells

The three current methods for differentiation of hPSC into CMs are by embryoid body formation (EB), monolayer culture and inductive co-culture. The first protocols used to develop CMs from EBs had difficulties with reproducibility due to EB sizes, phenotypic "drift" in the undifferentiated cell population dependent on basal culture conditions and unidentified components such as serum [11]. The first improvements in developing CMs from EBs therefore focused on defined culture conditions and EB sizes. The culture media including serum is replaced by a serum-free defined media for cardiac differentiation, like the commercially available media APEL (stem Cell Technologies, Vancouver, BC, Canada) and StemPro-34 (Invitrogen) [69, 70]. To still be able to induce cardiogenesis, several growth factors can be added which are normally present in human cardiac development. A study using a genetically tagged HES3 cell line in which MIXL1, a primitive streak marker, is linked to green fluorescence protein (GFP) showed the highest mesoderm differentiation of cells in day 3 EBs when adding the growth factors BMP4 and/or activin A to the APEL medium [71]. To induce cardiac lineages from mesodermal progenitors the concentration and the time point at which the growth factors are added is important. Moreover, for some growth factors it is important to inhibit signaling at a later stage, such as Wnt [72, 73]. Furthermore, the addition of vascular endothelial growth factor can improve the development of CMs from EBs [69].

Several EB protocols can be used to develop CMs, such as spin EBs, Microwell EBs and Micropatterned EBs [detailed review, see [11]]. Spin EB protocols were initially directed at overcoming the problem of the heterogeneity of EB size formed in early studies. In spin EB protocols, centrifugation causes the undifferentiated cells to form aggregates of identical size into low attachment U- or V-bottomed wells. However, enzymatic adaptation with polyvinyl alcohol is essential for initial aggregation of the hESCs . These spin EBs are then differentiated using APEL medium enriched with GFs [11, 70]. Furthermore, several studies reported generation of CMs from spin EBs for several hPSC lines by solely using small molecule modulation of regulatory elements of Wnt/β-catenin signaling [73-75]. Moreover, β-catenin is reported to be required in hPSC differentiation to mesoderm and cardiac progenitors induced by Gsk3 inhibitors [74].

Microwell EBs are generated in a plate with defined microwells of which the bottom is coated with Matrigel extracellular matrix (ECM) to stimulate adhesion. The hESC aggregates generated in these wells are of defined sizes and grow efficiently. The hESC aggregates can be further used for standardized EB-mediated differentiation, similar to spin EBs. A drawback of this microwell technology is that it is not commercially available and requires the knowledge to, and equipment for, microengineering the microwells [11].

A third EB protocol that can be used is based on micropatterning. Micropatterned EBs are creat-

ed by plating size-specified hESC colonies from single-cell suspensions onto micropatterned ECM islands [76, 77]. This technique creates size-controlled EBs with optimal size range for maximizing mesoderm formation and cardiac induction.

Different than obtaining CMs from EBs, is the monolayer differentiation approach by which first a relatively uniform monolayer of hPSC is grown. The monolayer of hPSC can be differentiated within the same plate, in contrast to EBs that need to be replated before differentiation. Without the complex barriers present in EBs, application of growth factors (GF) and other interventions is more controllable in monolayer differentiation. The differentiation approach can either be done with a simple monolayer or with a matrix sandwich, in which a layer of Matrigel mixed with medium and/or GF is put on top of the monolayer of hPSC, increasing the purity of CMs [78].

The last approach to derive CMs from hPSCs is by coculturing with visceral endoderm-like cells. Visceral endoderm is essential in the developing embryo concerning the differentiation of cardiogenic precursor cells. Coculturing with visceral endoderm-like cells and with serum- and insulin-free medium, resulted in beating colonies containing ≈25% CMs [79]. It is a simple, rapid technique requiring very few cells and sufficient numbers of CMs are generated to visually detect beating.

The differentiation of hPSCs into CMs is intended to be controlled in a similar way as in embryos in which well-timed activation of TFs drive the formation of the nascent-precardiac mesoderm and determine cell fate. All protocols to differentiate hPSCs to CMs involve 6 similar steps (figure 1). The first step is the transition from hPSC to a mesoderm progenitor cell (day 0), otherwise known as the epithelial-mesenchymal transition [11, 53]. Transcription factors involved in the self-renewal of hPSCs and/or preventing differentiation are FGF2, Oct4, Nanog and Sox2 [11, 53]. The second step from mesodermal progenitor cells to precardiac mesoderm cells (day 2) can be induced by four major signaling pathways; BMP, TGFβ/Activin/Nodal, Wnt and FGF, with very specific temporal windows for effectiveness [53]. Furthermore, removal of insulin (until day 4, cardiac mesoderm development) is beneficial to the differentiation efficiency [80]. The markers characteristically expressed by precardiac mesodermal cells are Brachyury (T) and MIXL1 [11, 53]. The third step is the specification of the precardiac mesoderm to the cardiac mesoderm (day 3-4). The markers expressed at this stage are Mesp1 and the surface markers KDR and PFDRα [11, 53]. The fourth step is cardiac specification in which the cardiac mesoderm further develops into cardiac progenitor cells (day 5-6). Transcription markers differ per protocol but commonly include DKK1 [69, 81], or other factors inhibiting Wnt signaling; factors inhibiting BMP, like Noggin [82]; and factors inhibiting TGFβ1 and Nodal [53, 81]. The major markers involved are Isl1, Nkx2-5, GATA-4, Tbx5, Tbx20 and Mef2C [11, 53]. The fifth step involves cardiomyocyte differentiation of the cardiac progenitor cell into the immature cardiomyocyte (day 7+), including expression of the markers Nkx2-5, GATA4 and surface markers SIRPA and VECAM-1 [11, 53]. Furthermore, IGF1 and IGF2 can enhance proliferation of hPSC-CMs [83]. The final step is the maturation of immature CMs to mature CMs concerning electrophysiology, Ca^{2+} handling, myofibril organization and sarcomeric stirations [53].

Protocols for differentiation of hPSCs have dramatically improved concerning reproducibility in EB size and culturing media. Still, reproducibility is a concern regarding variability when the same hPSC lines and/or protocols are used [84, 85]. This interline variability can be caused by differences in the initial state of the hPSCs [31, 32], conditions used to maintain lines, epigenetic status regarding origin of tissue of hiPSCs and intrinsic differences in endogenous growth factor production between the different lines [11, 53, 81, 85-87].

Electrophysiology and contractility of human pluripotent stem cell derived cardiomyocytes

The cardiac action potential (AP) serves a major role in cardiac electrical and contractile function [88]. APs are recorded in single cells and clusters in both hiPSC-CMs and hESC-CMs. Both sources exhibit the three AP phenotypes (nodal-, atrial- and ventricular-like) as found in the human adult heart [13, 79, 89-92]. The criteria used to select a specific AP phenotype varies among studies, but usually is based on qualitative or quantitative combination of the maximum diastolic potential (MDP), upstroke velocity (dV/dtmax), action potential duration (APD), action potential amplitude (APA), beating rate (BR), and phase 4 (diastolic) depolarization rate (DDR) [93]. For example, cells with more negative MDP, longer APD, lower BR, higher dV/dtmax, larger APA, and lower DDR may be considered to be ventricular-like [93]. In both hPSC-EBs, usually one AP phenotype predominates within one EB [89, 91, 92]. In general, AP properties in hESC-CMs and hiPSC-CMs are those of immature cardiac cells (relatively positive MDP, low upstroke velocity, and diastolic depolarization) [88].

Ion currents: ventricular-like hPSC-CM compared to adult CM

Multiple studies have characterized the electrophysiological properties in both hESC-CM and hiPSC-CM. Gene expression of the major currents responsible for the AP in human ventricular CMs are functionally expressed and some increase with differentiation time [for review see [88]]. Below the findings of the major ion currents are discussed for hESC-CMs and hiPSC-CMs, and compared to adult ventricular CMs and shown in figure 2. Comparisons made between data from independent studies should be made carefully, due to potential differences in experimental conditions and voltage protocol applied [88].

Single cell patch-clamping studies revealed that the fast sodium current, I_{Na}, has a similar peak amplitude in hESC-CM (244 pA/pF at 0 mV [94]) and hiPSC-CM (216 pA/pF at -20 mV [95]), compared to the estimations made from the O'Hara computational model for human adult ventricular CM (196 pA/pF at -17 mV [96]). Moreover, one study shows that I_{Na} increases fivefold in magnitude from 1 to 8 months of differentiation in hESC-CM obtained from END-2 cocultures [10]. Moreover, in hESC-CM from END-2 cocultures gene expression of SCN5A and SCN1B increased substantially between 1 and 8 months of development [10]. In hiPSC-CM it is expressed only in differentiated hiPSC-CMs, not in undifferentiated cells [97]. Satin et al 2007 suggested a fetal phenotype based on a shift in Na^+ channel activation kinetics which we confirmed in our own study [8].

Peak L-type Ca^{2+} current, $I_{Ca,L}$, densities are measured in single ventricular hESC-CMs via voltage clamping [8-10, 98]. Current densities range from 2.2 pA/pF at 10 mV at 47 days of differentiation [98] to 10 pA/pF at -10 mV at 8 months of differentiation [10], suggesting an increase in $I_{Ca,L}$ magnitude over time. This is in line with the two studies reporting $I_{Ca,L}$ current densities measured at different time points during differentiation [9, 10]. Current densities of peak $I_{Ca,L}$ measured in hiPSC-CM of 30-50 days old are higher (17 pA/pFat 0mV [95]), compared to hESC-CM of similar age (4.3 pA/pF at 0 mV [8]). In human adult ventricular CM peak $I_{Ca,L}$ (10.2 pA/pF at 5 mV [99]) are similar to hESC-CMs at 8 months of differentiation [10]. The later study also found an up regulation in gene expression of CNCNB2, encoding for $Ca_v\beta2$, at 8 months after differentiation of hESC-CM from embryonic bodies, compared to 1 months after differentiation. CACN1C, encoding for the protein $Ca_v1.2$, is not expressed in undifferentiated cells, but is present in differentiated hESC-CMs at 8-15 days after differentiation from END-2 co-cultures [79]. hESC-CM obtained from embryonic bodies express CACN1C constant throughout differentiation. Moreover, the protein $Ca_v1.1$, encoded from the CACNA1S can be up regulated by miR-1 transduction in hESC-CM from embryonic bodies with activin A and BMP growth factor treatment [90]. In hiPSC-CM, CAC-

NA1C is only expressed in differentiated cells if they originated from Dermal 201B7 cell lines [97]. If originated from hFib2 cell lines, a significant increase over the first 21 days of differentiation can be measured [100]. hiPSC-CM from cell lines made from foreskin or hFib2 show a significant up-regulation over the first 21 days of differentiation in CACNA1D, encoding the $Ca_v1.3$ protein [100, 101].

Voltage clamp studies measuring the transient outward current, I_{to}, show a lower current density in hiPSC-CM (2.5 pA/pF at 60 mV [95]) then in hESC-CM (6 pA/pF at 50 mV [90]), but within the range of measurements performed on adult ventricular cells (2.3 to 7.9 pA/pF at 60 mV [102]).

One study reports that the KCND3 gene, encoding for the $K_v4.3$ protein, is not present in undifferentiated cells from hESC-CM END-2 coculture, but becomes expressed in differentiated cells of 8-15 days old [79]. However, another study did report that the KCND3 gene is already present in undifferentiated cells from suspended embryonic bodies [9]. The same study also found the KCNA4 gene, encoding for the $K_v1.4$ protein, to be present in suspended embryonic bodies after 25 days of differentiation. Moreover, KCNA4 expression reported to be higher at 8 months than at 1 month of differentiation from END-2 coculture [10] and can be up-regulated by MiR-1 transduction in hESC-CM from embryonic bodies

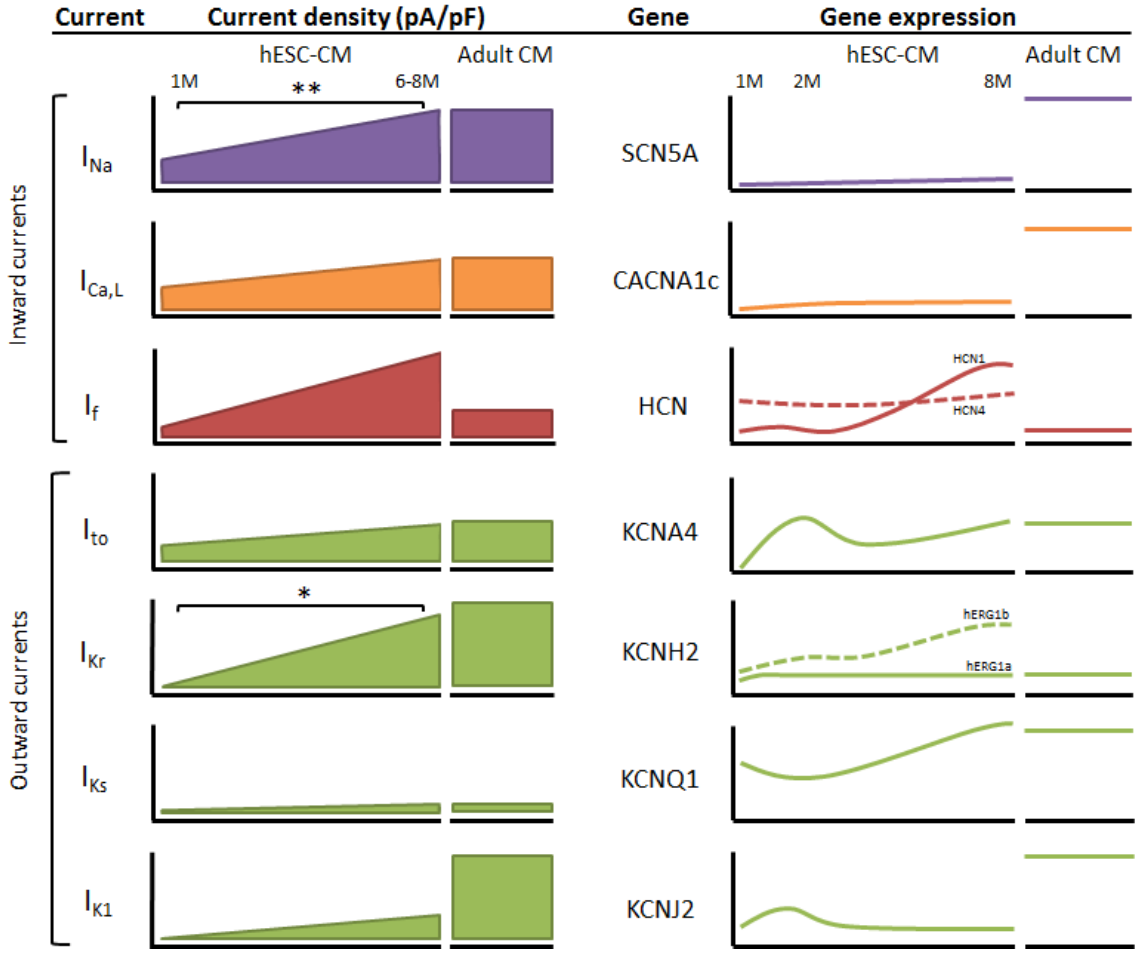

Figure 2. Electrophysiological maturation of human embryonic stem cell derived cardiomyocytes (hESC-CM) compared with human adult CMs, including current density and gene expression of the major channel subunit measured at different culture time points.
* p-value of < 0.05
** p-value of < 0.01
NOTE: current density of I_{K1} was only measured in 1 out of 3 cells.

with activin A and BMP growth factor treatment [90]. One study reported that the KCND3 gene is also present in hiPSC-CM, however regarding this gene, no maturation effects are reported [103]. The slow delayed rectifier current, I_{Ks}, is recorded as a chromanol 293B sensitive current in hPSC-CMs [10, 90, 104]. I_{Ks} is a very small current making detection difficult, but when measured, the tail current in hESC-CM is 0.7 pA/pF at -40 mV[104, 105] and in hiPSC-CM 0.31 pA/pF at 40 mV (5 out of 16 cells [95]. From the O'Hara model, I_{Ks} could be estimated in human adult ventricular CM and had a magnitude of 0.58 pA/pF at -40 mV under physiological conditions[96]. Current densities remained relatively constant during differentiation in hESC-CM from END-2 co-cultures. However gene expression of KCNQ1, encoding for the protein K_v7.1, shows to be slightly higher at 8 months of differentiation compared to at 1 months after differentiation [10]. Another report shows that it is expressed in undifferentiated cells, then expression decreases at mid-stage and increases again at late-stages after differentiation [79] of hESC-CM from END-2 co-cultures. KCNQ1 in hiPSC-CM derived from hFib2 cell lines is only expressed in differentiated hiPSC-CMs [106].

The rapid delayed rectifier current, I_{Kr}, is recorded as a E-4031 sensitive current in both hPSC-CMs [8, 95]. The maximal current density is about the same in hESC-CM (0.4-0.7 pA/pF at 10 mV [90, 105]), hiPSC-CM (0.4 pA/pF at -10 mV [95] and human adult ventricular CM estimated from the O'Hara model (0.82 pA/pF at 7 mV [96]). However, the maximal current measured in hiPSC-CM occurs at a more negative voltage [90, 105]. Moreover, I_{Kr} channels are able to conduct cesium (Cs^+) currents, which has the benefit to be only permeated by I_{Kr}. Measurements performed in hESC-CM by Cs^+ permeation showed a well-detectable tail current density of −11.5 pA/pF at 50 mV [8]. The current density in hESC-CM reported to be about 70-fold higher after 8 months of differentiation, compared to 1 month [10]. The KCNH2 gene encodes for the protein K_v11.1, the α subunit of this potassium ion channel, also known as the hERG channel. The hERG channel has several isoforms. hERG1a in hESC-CMs obtained from suspended embryonic bodies appeared to be always present [9], is increased at 55 days after differentiation compared to 8, 20 and 30 days [107] and can be up-regulated by miR-1 transduction [90]. However, in hESC-CM hERG1b is only present after day 16 [9] and is higher after 8 months of differentiation than after 1 month [10]. Gene expression of KCNE2, encoding for MiRP1, also is the highest as compared to 8, 20 or 30 days after differentiation into hESC-CM [107]. In hiPSC-CM, KCNH2 gene expression is only expressed in differentiated hiPSC-CMs if obtained from dermal 201B7 cell lines. If obtained from foreskin or hFib2 KCNH2 gene expression becomes up-regulated significantly over the first 21 days of differentiation [101, 106].

The inward rectifier potassium current, I_{K1}, is very hard to detect in both hPSC-CM [8, 94]. One study reported a significant increase from 2 (0.9 pA/pF) to 8 (2.57pA/pF) months of differentiation in hESC-CM [10]. However, current densities measured in human adult ventricular cardiomyocytes remain to be the highest (10 pA/pF at -90 mV [108]). Gene expression of KCNJ2, encoding for the protein KiR2.1, in hESC-CM is always present and stops increasing by day 57 [9]. Another study confirmed a higher gene expression of KCNJ2 at 8 months, compared to 1 month after differentiation [10]. Moreover, KCNJ2 gene expression can be up regulated by miR-1 transduction [90]. In hiPSC-CM derived from Dermal 201B7, expression of KCNJ2 is present, but no maturation effects are reported [103]. Still, compared to adult CM, expression levels are very low.

Measurements of the pacemaker current, I_f, in single hESC-CM via voltage clamping showed to have a magnitude peak ranging from 4 pA/pF to about 10 pA/pF at -120 mV [8, 10, 90, 94], with the peak magnitude of hiPSC-CM within that range (4.1 pA/pF at -120 mV [95]). The peak magnitude of I_f in human adult ventricular CMs is much smaller (1.9 pA/pF at -120 mV [109]). Moreover, I_f cur-

rent density closely resemble current densities seen in nodal cells and play a major role in the spontaneous activity of hESC-CM [8]. Gene expression of HCN1 is not present at days 5 and 10 of differentiation but is present at days 15 and 20 and further increases in hESC-CM until day 25 of differentiation. After that time-point, expression decreases at days 57 and 110 [9, 110], which is in line with electrophysiological measurements showing a down regulation of I_f during differentiation [9]. Expression of HCN2 is present at days 15 and 20 of differentiation and remains relatively constant [9, 110]. HCN4 gene expression decreases after miR-1 transduction and with differentiation time [9, 90, 110]. In hiPSC-CM originated from foreskin and hFib2 cell lines, HCN2 expression became significantly upregulated in the first 21 days of differentiation [101, 106].

Contractility

Besides the somewhat immature electrical phenotype of both hPSC-CMs compared to human adult CMs, there are also differences concerning contractility, in specific Ca^{2+} handling.

Various studies of hESC-CMs and hiPSC-CMs have demonstrated the presence of structural and contractile proteins, like cross-striations, troponin, myosin heavy chain, tropomyosin and α-actinin [10, 13, 14, 16, 79, 89, 98, 103, 106, 111-115]. Mechanical contraction is measured in human embryonic bodies (hEBs), single hESC-CMs and single hiPSC-CMs [14, 115]. Adding isoproterenol increased rate of contraction in both hESC-CM and hiPSC-CM, with a larger increase in contraction in the later following addition of isoproterenol in cells of 18-70 days old compared to 10-15 days old cells [103, 115]. Other studies investigating rate- and time dependent changes in contractions showed that the SR Ca^{2+} stores did not contribute to contraction. Furthermore, adult myocardium characteristically shows a positive force-frequency relation [116] whereas small aggregates of hiPSC-CMs and hEBs and isolated hESC-CMs have a negative force-frequency relation [14, 112, 115]. Adult myocardium shows post-rest potentiation of contraction, due to an increase during the rest period of SR Ca^{2+} stores. In contrast, several studies showed no post-rest potentiation in hEBs and isolated hESC-CMs and a weak post-rest potentiation in small aggregates of hiPSC-CM [14, 112, 115]. Conversely, tissue constructs of hESC-CMs do show force-length relations with a positive slope, similar to the adult heart [117].

Calcium cycling

Many studies have put effort in characterizing Ca^{2+} cycling, including key Ca^{2+} cycling proteins and/or gene products, Ca^{2+} transients, Ca^{2+} release and Ca^{2+} cycling processes in hESC-CM and hiPSC-CM.

Single hESC-CM showed to have comparable mRNA levels of $Ca_v1.2$ compared to human adult CM [12]. In hiPSC-CM originated from Fetal 201B7 and hFib2 cell lines, $Ca_v1.2$ is expressed at the mRNA level, but no numbers or comparisons are given [97, 106]. Compared to human adult CM, mRNA levels of ryanodine receptor 2 (RyR2) showed to be about 4-fold lower in hESC-CM clusters [118], about a 1000-fold lower in single hESC-CM [12] and even lower in hiPSC-CM [13]. Protein expression of SERCA2a is significantly lower in hiPSC-CMs compared to hESC-CM [13]. Moreover, SERCA2a expression in hESC-CM (hEBs) reported to be similar compared to adult CM [14, 112]. SERCA2a expression in single hESC-CM is similar to levels reported in fetal ventricular myocytes, but much lower compared to adult ventricular myocytes [114]. Reversely, NCX expression in hESC-CM resembled adult CMs more than fetal CMs [114]. Expression in hiPSC-CM reported to be lower compared to hESC-CMs [13]. Calsequestrin (CSQ) in hESC-CM originated from H9.2, I3 (hEBs) and H1, HES-2 (single cells) is not expressed [14, 112, 114]. However, in hESC-CM originated from H7, HES-3 (single cells) and H9.2, SA002 (hEBs/clusters) CSQ expression is found [115, 118], with an 70-fold increase in mRNA expression in hPSC-CM clusters compared to human adult heart [118]. Expression of CSQ reported to be expressed in hiPSC-

CM in both hEBs and single cells [13, 16, 115]. Phospholamban is not expressed in hESC-CM [14, 112] and also the regulatory protein triadin reported to be absent [114].

Functional Ca^{2+} transients are reported in hESC-CM (hEBs, engrafted hESC-CMs, small cell aggregates and single cells) [14, 79, 98, 111-114] and hiPSC-CMs [13]. However, some findings suggest that Ca^{2+} cycling in hESC-CMs and hiPSC-CMs is functional, but rather immature [88, 93]. Several studies reported a difference in the functional properties of intracellular Ca^{2+} handling of hESC-CM compared to adult CM, likely caused by the immature capacity of the sarcoplasmic reticulum (SR) [14, 112]. [Reviewed by [88, 93]].

Furthermore, Ca^{2+} waves are measured along the transverse axis of hESC-CM and hiPSC-CM and in both U-shaped waves are found, indicating the absence or immaturity of T-tubules [13, 94]. The delay of Ca^{2+} release is found to be greater in hiPSC-CM [13]. Electron microscopy revealed some developing T-tubules associated with the SR in hESC-CM [111]. However, in hiPSC-CM no T-tubules can be found [119], although the specific study also found poorly developed SR compared to other studies.

There is conflicting evidence regarding the degree of maturity concerning Ca^{2+} cycling. Some report immature aspects of Ca^{2+} cycling, regarding $I_{Na/Ca}$ driven Ca^{2+} transients in hESC-CM, which is characteristically high in fetal CMs and low in adult CMs [98]. Additionally, release of Ca^{2+} stores mediated by IP3R pathway activation is also high in hESC-CM and low in adult CM [120]. Moreover, blocking RyR release or SERCA uptake did not affect Ca^{2+} transients in hESC-CMs, indicating that SR Ca^{2+} stores are not functional and that mainly transarcolemmal Ca^{2+} flux causes Ca^{2+} transients and contractions [14]. Contradictory, others report mature aspects of Ca^{2+} cycling, concerning the finding of a close co-localization in hESC-CM of RyR and L-type Ca^{2+} channels and a “gain” in excitation and contraction coupling at increasing negative potentials, characteristically to adult myocytes [121, 122], suggesting mature Ca^{2+} cycling in single hESC-CMs. Furthermore, both hESC-CM and hiPSC-CM release Ca^{2+} from the SR following large doses of caffeine, revealing the presence of functional SR Ca^{2+} stores [13, 16, 119]. Recently, a study reported that SR Ca^{2+} stores regulated the spontaneous beating of hiPSC-CMs and blocking Ca^{2+} release via either RyR or IP3R decreased the rate of spontaneous beating, similar to findings in hESC-CMs [16, 88, 123] [Reviews on Ca^{2+} cycling in hPSC-CMs: [15, 124]].

Discussion and Conclusion

In this review, a description is given about which transcription factors and signaling pathways play a role in human fetal development of the heart. Moreover, different protocols for cardiac differentiation of hPSC into CMs are described. During early heart development in humans three major signaling pathways are involved, e.g. FGF, BMP and Wnt. Moreover, factors of the fourth major signaling pathways, TGFβ/Nodal/Activin, are triggered by shh during early heart development, facilitating the left-right asymmetry of the human heart. To mimic fetal cardiac development, the same major signaling pathways are used during mesodermal development in cardiac differentiation protocols for hPSC-CMs. Once the mesoderm has fully developed into cardiac mesoderm during *in vitro* differentiation, these signaling pathways are inhibited by DKK1, Noggin, Dorsomorphin and SB431542 (TGF-βR inhibitor). During *in vitro* cardiac differentiation of hPSC, the cardiac progenitor cell expresses similar transcription factors compared to *in vivo* heart tube formation. The immature cardiomyocyte expresses similar transcription factors in both *in vivo* and *in vitro* cardiac development. However, during heart tube formation, the immature cardiomyocyte expresses two other major transcription factors, namely Tbx5 and Mef2c. These transcription factors at this stage of development are involved in interpretation of the patterning signals within the primitive heart tube.

During *in vitro* cardiac differentiation of hPSC, no heart tube is formed and therefore might be lacking the expression of these two factors.

Unfortunately, data on ion channel expression in human fetal cardiomyocytes is lacking. This lack of data makes it difficult to compare ion channel expression of hPSC-CM to human fetal cardiomyocytes, with the exception of the α1c subunit of L-type Ca^{2+} channels. The mRNA levels of the α1c subunit increased during human heart development with a maximum at the adult stage. In hESC-CM, a similar increase in gene expression of the α1c subunit can be measured from 1M up to 8M of differentiation. Moreover, ion current recordings of $I_{Ca,L}$ in hPSC-CM show similar current densities as measured in human adult CMs. These findings suggest an mature phenotype regarding L-type Ca^{2+} channels in hPSC-CM similar to human heart development. Regarding ion current densities in hPSC-CM, two ion currents have an immature phenotype; I_{K1} and I_f. Maturation of I_{K1} and I_f in hPSC-CM will improve the electrophysiological maturation and thereby improving the applicability of hPSC-CM as an new *in vitro* model.

An immature phenotype is also observed in hPSC-CM concerning contractility and Ca^{2+} cycling. One well studied Ca^{2+} cycling protein is SERCA2a. During human cardiac development mRNA expression of SERCA2a does not significantly change from fetal to the adult hearts. However, protein levels of SERCA2a do increase during human development, suggesting a difference in posttranslational processing. Expression of SERCA2a protein levels in single hESC-CMs show to be similar to the fetal stage in human heart development. Whereas, expression in hEBs reported to be comparable to adult CMs. Besides differences in SERCA2a expression between human fetal and adult cardiomyocytes, differential expression of L-type Ca^{2+} channel α1c subunit mRNA levels and more NCX mRNA and protein levels in fetal compared to adult CMs suggesting an alternate fetal Ca^{2+} handling compared to the human adult heart.

An attempt is made to relate signaling pathways and transcription factors involved during human cardiac development to ion channel expression. Thereby, improving cardiac differentiation protocols of hPSC to obtain hPSC-CMs with an improved electrophysiological maturation. Signaling pathways and transcription factors are described during human cardiac development and in cardiac differentiation protocols and electrophysiological maturation in hPSC-CM is reviewed. However, the lack of data regarding electrophysiological maturation during human fetal cardiac development obstructs the establishment of relationships between signaling pathways during human cardiac development and ion channel expression.

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