Chapter 2
Embryonic Stem Cells for Cardiac Regeneration

Francesco Moccia, Federica Diofano, Paola Rebuzzini, and Estella Zuccolo

2.1 Introduction

Cardiovascular disease (CD) is the leading cause of death and morbidity in developed countries. Heart failure (HF) is the common end point of virtually all cardiac disorders, including acute myocardial infarction (AMI), coronary artery disease, hypertension, stroke, and diabetes, and affects about 1–2 % of the population in the Western world. The incidence of HF dramatically increases in ageing subjects and, consequently, represents the most frequent cause of hospitalization for those aged 65 and over (Pfister et al. 2014). Unlike smooth and skeletal muscle cells, adult cardiac myocytes show a limited regenerative capacity and cannot restore ventricular function to supply sufficient blood flow to the body (Jakob and Landmesser 2013; Pfister et al. 2014; Moccia et al. 2014). Heart transplantation (HT) represents the ultimate approach to treat end-stage HF, but this therapy is invasive, expensive and not suitable for elderly patients affected by comorbidities. Moreover, donor organ shortage is a major limitation factor worldwide and causes the death of an increasing number of subjects with head-stage HF who are listed for HT. Cell based therapy (CBT) provides an alternative option to replace damaged myocardium and vasculature and restore cardiac contractility (Moccia et al. 2013, 2014; Jakob and Landmesser 2013; Pfister et al. 2014). Several cardiac and non-cardiac stem/precursor/progenitor cells have been used in both pre-clinical studies and clinical trials to regenerate the failing heart, including bone marrow-derived mononuclear cells (MNCs), skeletal myoblasts, mesenchymal stem cells (MSCs), endothelial progenitor cells (EPCs), and

F. Moccia (✉) • F. Diofano • E. Zuccolo
Laboratory of General Physiology, Department of Biology and Biotechnology "Lazzaro Spallanzani", University of Pavia, via Forlanini 6, Pavia 27100, Italy
e-mail: francesco.moccia@unipv.it

P. Rebuzzini
Laboratory of Developmental Biology, Department of Biology and Biotechnology "Lazzaro Spallanzani", University of Pavia, via Forlanini 6, Pavia, 27100, Italy

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cardiac progenitor cells (CPCs) (Moccia et al. 2013, 2014; Jakob and Landmesser 2013; Pfister et al. 2014). Unfortunately, there is no evidence that these cell populations, even when harvested from patients' heart, such as CPCs, engraft within damaged myocardium, acquire a contractile phenotype and behave as genuine myocytes. Moreover, most of these studies did not show any remarkable improvement of cardiac performance: they recorded a modest 5 % increase, if any, in left ventricular ejection fraction (LVEF) of recipient hearts, which was mainly due to the paracrine release of factors and inhibit apoptosis, stimulate local angiogenesis and extracellular matrix remodelling, and recruit endogenous CPCs (Parsons 2012; Menasché 2012). This led to a paradigmatic shift in the field, according to which CBT should rather be carried out by using cardiac lineage-committed stem cells that are physically capable of replacing lost native CMs and functionally integrating within the beating heart. This novel context has led to the re-appreciation of the therapeutic use of pluripotent embryonic stem cells (ESCs), unspecialized cells which can be indefinitely cultivated in vitro, maintaining their features (undifferentiated status and self-renewal) and their intrinsic ability to generate all the terminal differentiated cell types, including cardiomyocytes (CMs). Therefore, ESCs hold tremendous potential for treating human disorders featuring tissue loss and insufficiency, such as HF and AMI (Wong and Bernstein 2010; Parsons 2012; Menasché 2012). Accordingly, both murine and human ESCs (mESCs and hESCs, respectively) differentiate into contracting myocytes that can integrate within damaged myocardium when properly manipulated in vitro (Kehat et al. 2001, 2004; Boheler et al. 2002; Passier et al. 2008). The present chapter aims at addressing the basic issues regarding isolation, characterization and CM differentiation of mESCs and hESCs and at focusing on their advantages and disadvantages for cardiac regeneration (Tables 2.1 and 2.2).

2.2 Murine Embryonic Stem Cells Isolation, Characterization and CM Differentiation

Mouse ESCs are undifferentiated, self-renewing and undifferentiated cells, derived from the inner cell mass (ICM or epiblast) of a blastocyst (Evans and Kaufman 1981; Martin 1981). Blastocysts, obtained or by natural (4 days *post coitum*) or by in vitro fertilization, are singly plated on plates coated with a feeder layer of immortalized or embryonic mouse fibroblasts and with an appropriate culture medium containing the leukemia inhibitory factor (LIF), necessary to repress mESC differentiation. To date, the presence of the feeder layer is necessary because of its ability to release the trophic factor, whereas LIF, a soluble glycoprotein belonging to the interleukin (IL)-6 family, is fundamental for mimicking the in vivo conditions in which it is released by the trophoectoderm cells and is perceived by the ICM cells, where it triggers the signal transduction finally activating the STAT3 transcription factor. STAT3 in turn transcribes all those genes involved in the maintenance of the undifferentiated state (Smith et al. 1988; Wobus and Boheler 2005; Graf et al. 2011).

After 2–4 days of culture, the rupture of the zona pellucida enables the outgrowth of both ICM cells and trophoblast cells, which are readily identifiable by phase-contrast

Table 2.1 Approaches to enhance cardiomyocyte yield from embryonic stem cells (ESCs)

Approach	Comments	References
Growth factors	Retinoic acid enhanced cardiomyocyte differentiation in mouse ESCs	Wobus et al. (1997)
	Exogenous glucose, amino acids, vitamins, and selenium enhanced cardiomyocyte differentiation in mouse ESCs	Guan et al. (1999)
	LIF enhances and inhibits cardiomyocyte commitment and proliferation in mouse ESCs in a developmental stage-dependent manner	Bader et al. (2000)
	Reactive oxygen species enhanced cardiomyocyte differentiation in mouse ESCs	Sauer et al. (2000), Buggisch et al. (2007), Sharifpanah et al. (2008), Wo et al. (2008)
	Endoderm enhanced cardiomyocyte differentiation in mouse ESCs	Bader et al. (2001), Rudy-Reil and Lough (2004)
	A TGF/BMP paracrine pathway enhanced cardiomyocyte differentiation in mouse ESCs	Behfar et al. (2002)
	Activation of the MEK/ERK pathway enhanced cardiomyocyte differentiation in mouse ESCs	Kim et al. (2007)
	Verapamil and cyclosporine enhanced cardiomyocyte differentiation in mouse ESCs	Sachinidis et al. (2006)
	5-Aza-2¢-deoxycytidine enhanced cardiomyocyte differentiation in human ESCs	Xu et al. (2002)
	Endoderm cell lines enhanced cardiomyocyte differentiation in human ESCs	Mummery et al. (2003, 2007)
	Ascorbic acid enhanced cardiomyocyte differentiation in human ESCs	Takahashi et al. (2003)
	Directed differentiation with activin A and BMP4 in monolayers of human ESC	Laflamme et al. (2007)
Genetic engineering	Lineage-restricted drug resistance gene resulted in highly purified cardiomyocyte cultures from mouse ESCs	Klug et al. (1996), Zandstra et al. (2003), Schroeder et al. (2005)
	Highly purified cardiomyocyte cultures generated by FACS of mouse ESCs expressing a lineage-restricted EGFP reporter	Müller et al. (2000)
	Targeted expression of a-1,3-fucosyltransferase enhanced cardiomyocyte differentiation in mouse ESCs	Sudou et al. (1997)
	Coexpression of EA1, dominant negative p53, and dominant negative CUL7 enhanced cell cycle in mouse ESC-derived cardiomyocytes	Pasumarthi et al. (2001)
	Expression of SV40 T antigen enhanced cell cycle in mouse ESC-derived cardiomyocytes	Huh et al. (2001)
	Antagonization of Wnt/b-catenin enhanced cardiomyocyte differentiation in mouse ESCs	Singh et al. (2007)
	Lineage-restricted drug resistance gene resulted in highly purified cardiomyocyte cultures from human ESCs	Anderson et al. (2007), Xu et al. (2008b)

(continued)

Table 2.1 (continued)

Approach	Comments	References
Miscellaneous	A single 90-s electrical pulse applied to day 4 EBs increased cardiomyocyte differentiation in mouse ESCs	Sauer et al. (1999)
	Application of mechanical loading enhanced cardiomyocyte differentiation in mouse ESCs	Shimko and Claycomb (2008), Gwak et al. (2008)
	FACS for transient Flk-1 isolated cardiomyogenic progenitors from mouse ESCs	Kattman et al. (2006)
	Cardiomyocyte enrichment using density centrifugation and cultures of cell aggregates in human ESCs	Xu et al. (2006)
	Activin A, BMP4, bFGF, VEGF, and DKK1 treatment, followed by KDR+	Yang et al. (2008)
	/c-kit–FACS, identified cardiovascular progenitor cells in human ESCs	

LIF leukemia inhibitory factor, *TGF* transforming growth factor, *BMP* bone morphogenetic protein, *MEK* mitogen-activated protein kinase, *ERK* extracellular-signal-regulated kinase, *FACS* fluorescence-activated cell sorting, *EGFP* enhanced green fluorescent protein, *EBs* embryoid bodies, *bFGF* basic fibroblast growth factor, *VEGF* vascular endothelial growth factor

microscopy. Clusters of ICM cells are thus mechanically isolated, dispersed and replated onto MEF feeder layers until clonal mESC line is established. In the presence of the feeder layer and LIF, mESCs may be cultured for a long time maintaining their unlimited proliferation capacity, pluripotency and ability to differentiate into all cell lineages (Hescheler et al. 1997; Boheler et al. 2002; Wobus and Boheler 2005). This is consistent with their ability to create teratomas comprising multiple cell types from the three germ layers upon injection into immunodeficient mice (Passier et al. 2006). It should, however, be pointed out that, unlike earlier predictions (Boheler et al. 2002), recent studies have demonstrated that (sub)chromosomal aberrations occur during mESC cultures (Neri et al. 2007; Rebuzzini et al. 2007; Gaztelumendi and Nogués 2014). Undifferentiated mESCs are characterized by a panel of cell surface and molecular markers that define their stemness; these include specific membrane antigens (SSEA-1,3) and membrane-bound receptors (gp130) and specific enzyme activities, such as telomerase and alkaline phosphatase (ALP). The transcriptional circuit responsible for self-renewal and pluripotency is governed by the epiblast/germ cell-restricted transcription factor Oct-3/4, whose expression levels must be maintained within an appropriate range to maintain stem cell self-renewal. A small elevation in Oct-3/4 levels (<2-fold) causes ESC differentiation into primitive endoderm or mesoderm, while suppressing Oct-3/4 expression induces them to acquire a trophectoderm phenotype. Oct-3/4 sustains self-renewal and pluripotency by acting in concert with Sox2, FoxD3, bone morphogenetic protein (BMP)-induced Smad-mediated activation of Id (inhibition of differentiation target genes), and LIF-dependent JAK2/Stat3 cascade. Additional molecular markers of pluripotency are represented by Nanog, Sox2 and Rex-1 that are expressed in the ICM, but are down-regulated during differentiation.

Table 2.2 Comparison of some properties of mouse and human embryonic stem cells

Marker	Mouse ES cells	Human ES cells	Reference
SSEA-1	+	–	Solter and Knowles (1978)
SSEA-3/-4	–	+	Thomson et al. (1998), Reubinoff et al. (2000), Xu et al. (2001), Henderson et al. (2002)
TRA-1-60/81	–	+	Thomson et al. (1998), Reubinoff et al. (2000), Xu et al. (2001), Henderson et al. (2002)
TRA-2-54	–	+	Henderson et al. (2002)
GCTM-2	–	+	Pera et al. (2000), Reubinoff et al. (2000)
TG 343	?	+	Henderson et al. (2002)
TG 30	?	+	Pera et al. (2000)
CD 9	+	+	Pera et al. (2000)
CD133/prominin	+	+	Kania et al. (2005), Carpenter et al. (2004)
Alkaline phosphatase	+	+	Wobus et al. (1984), Thomson et al. (1998)
Oct-4	+	+	Thomson et al. (1998), Pesce et al. (1999)
Nanog	+	+	Chambers et al. (2003), Mitsui et al. (2003)
Sox-2	+	+	Avilion et al. (2003), Ginis et al. (2004)
FGF4	+	–	Ginis et al. (2004)
LIF receptor	+	+/–	Richards et al. (2004)
Telomerase activity	+	+	Thomson et al. (1998), Armstrong et al. (2000)
Regulation of self-renewal	Via gp 130 receptors, MEF feeder layer, Nanog, BMP-4	Feeder cells (MEF or human cells), serum, bFGF, Matrigel	Niwa et al. (1998), Thomson et al. (1998), Xu et al. (2001), Chambers et al. (2003), Ying et al. (2003)
Growth characteristics in vitro	Tight, rounded, multilayer clusters	Flat, loose aggregates	Thomson et al. (1998)
EB formation	Simple and cystic EBs	Cystic EBs	Doetschman et al. (1985), Thomson et al. (1998), Itskovitz-Eldor et al. (2000)
Teratoma formation in vivo	+	+	Wobus et al. (1984), Thomson et al. (1998)

MEF mouse embryonic fibroblast, *EB* embryoid body

In vitro differentiation of mESCs requires the initial formation of 3D cell aggregates that have termed embryoid bodies (EBs), which may differentiate into into the three primordial embryonic germ layers. EBs are obtained by cultivating mESCs in hanging drops for 3 days and in suspension for further 2 days before being transferred onto gelatin-coated tissue culture dishes. The parameters that specifically influence the acquisition of cardiac phenotype are: (1) cell density in the EB, (2) culture medium, fetal bovine serum (FBS) or fetal calf serum (FCS), supplement with growth factors and other inducing agents (i.e. retinoic acid, DMSO, or co-colture with endoderm-like cells), (3) mESCs line, and (4) the time and duration of the differentiation process. Spontaneously, contracting cardiac myocytes become manifest between day 1 and 4 after plating between an outer epithelial layer and a basal layer of mesenchymal cells of the EB. The number of spontaneously beating foci and the rate of contraction rapidly augment through the differentiation process, but maturation (after about 1 month of culture) is accompanied by a reduction in average beating rate, as observed during normal cardiac development. Fully differentiated CMs normal fail to contract, but can be successfully maintained in culture for many weeks. Three stages can be distinguished during the differentiation process of murine EBs: early (pacemaker-like or primary myocardial-like cells), intermediate, and terminal (atrial-, ventricular-, nodal-, His-, and Purkinje-like cells) (Boheler et al. 2002; Becker et al. 2011). During early stages of differentiation, EBs-derived CMs (7 + 2 days) appear small and rounded, display sparse and irregularly organized myofibrils, or they can even lack them, and concentrate in round accumulations. At later stages (7 + 12 days), they organize into strands of elongated CMs endowed with mature sarcomeres and myofibrils (Hescheler et al. 1997; Boheler et al. 2002). Contracting myocytes are primarily mononuclear and rod-shaped cells, and exhibit cell-cell junctions resembling those that have been described in CMs of developing hearts. Densely packed and well organized myofibril bundles are evident at the terminal stages of differentiation, while sarcomeres show clear A bands, I bands, and Z disks. Likewise, intercalated disks, desmosomes, gap junctions, fascia adherens, and gap junctions have been detected, as well as the spreading of Lucifer yellow to adjoining cells after microinjection, which is indicative of functional gap junctions-mediated intercellular communication. Overall, the ultrastructural architecture, myofibrillar organization, and sarcomere length of mESCs-derived CMs are similar to those of foetal and neonatal rodent myocytes (Hescheler et al. 1997; Boheler et al. 2002).

CM differentiation of murine ESCs recapitulates the genetic program underlying cardiac development in mouse embryo in a developmentally regulated manner. The cardiac specific transcription factors Gata-4 and Nkx2.5 appear before transcripts encoding atrial natriuretic factor (ANF), myosin light chain (MLC)-2v, α-myosin heavy chain (α-MHC), β-myosin heavy chain (β-MHC), Na^{+}-Ca^{2+} exchanger and phospholamban. Similarly, sarcomeric proteins appear in the same time sequence as that described during cardiogenesis: titin (Z disk), α-actinin, myomesin, titin (M band), MHC, α-actin, cardiac troponin T, and M protein. Similar to foetal/neonatal CMs, early mESCs-derived CMs express slow skeletal muscle Troponin I and β-MHC rather α-MHC, which appear along with cardiac Troponin I in terminally

differentiated CMs, displaying more rapid contractions (Boheler et al. 2002; Wobus and Boheler 2005). Concerning their electrophysiological properties, early (7 + 2 days) and intermediate (7 + 5 − 8 days) mESCs-derived CMs display spontaneous depolarizations, resembling the pacemaker-like action potentials of the sino-atrial node of fully differentiated heart. On the other hand, terminally differentiated (7 + 9 − 18 days) cells present three major types of action potential: spontaneous sinusoidal, triggered ventricular- and atrial-like action potentials. Moreover, terminal, but not early, mESCs-derived CMs respond to β-adrenergic stimulation. Finally, while T tubules are normally absent in these cells, the mechanism of excitation-contraction coupling resemble those found in foetal/neonatal CMs by utilizing ryanodine receptors (RyRs) to trigger Ca^{2+} release in response to membrane depolarization (Hescheler et al. 1997; Boheler et al. 2002; Moccia et al. 2014). In particular, the role of RyR2 progressively increases during cardiac differentiation, while Ca^{2+} mobilization during the early phases of development also involves inositol-1,4,5-trisphosphate receptors ($InsP_3Rs$) (Fu et al. 2006).

2.3 Human Embryonic Stem Cells Isolation, Characterization and CM Differentiation

The technique exploited to harvest and cultivate mESCs was adapted to generate hESC lines from discarded pre-implanted embryos produced by IVF. Thomson and collaborators accomplished the derivation of the first human ESC line through immunosurgery (Thomson et al. 1998). Thomson's group cultured human embryos up to the blastocyst stage and applied the immunosurgery technique (Solter and Knowles 1978) using a rabbit anti-rhesus spleen cell antiserum, that targets the external trophoectoderm cells, surrounding the ICM, and is responsible for the activation of the guinea pig complement. The final result is the release of the ICM. The ICM was plated and maintained for 16 days in suspension and then was grown for 3 weeks in gelatin until the new line was established: they obtained the first human ESC line, called H9.

The basic features of hESCs resemble those of their murine counterparts, such as high telomerase activity and teratoma formation in immunodeficient mice. The transcriptional pluripotency hub represented by Oct-3/4, Sox2 and Nanog is conserved in hESCs, but their target different signalling pathways as compared to mESCs, as reviewed in Schnerch et al. (2010). Additionally, hESCs show different isoforms of stage-specific antigens (SSEA-3 and SSEA-4) and proteoglycans (TRA-1–60, TRA-1–81, GCTM-2) that are absent in mESCs (Wobus and Boheler 2005). Similar to mESCs, hESCs form teratomas and teratocarcinomas in immunodeficient in vivo and actively proliferate for long periods of time in culture, but display longer average population doublings (30–35 *vs.* 12–15 h) (Amit et al. 2000). LIF does not maintain pluripotency in hESCs, which must be therefore grown on feeder layers of MEFs or human tissues-derived cells supplemented with bFGF2. Nevertheless, it has been found that hESCs remain pluripotent when cultured on

Matrigel or laminin in the presence of MEFs-conditioned media (Xu et al. 2001), albeit the soluble factor responsible for self-renewal maintenance has not been identified. As discussed below, hESCs are now cultivated on MEFs without animal serum (Lee et al. 2005) but in the presence of specific serum-free defined media (serum replacement) (Mummery et al. 2012) to avoid any xenogenic contamination that could hamper their subsequent application on human subjects.

HESCs-derived cardiomyocytes were described 3 years after hESC identification (Thomson et al. 1998; Kehat et al. 2001). HESCs (cell line H9.2) were dispersed into small clusters (3–20 cells) by using collagenase IV and then grown in suspension with serum for 7–10 days to form EBs, which were then coated onto gelatine-coated plates. Spontaneous contractions started 4 days after plating (i.e. 11–14 days after the beginning of the differentiation process), while the highest number of beating areas was detected 20 days after plating (i.e. after 27–30 days of differentiation) (Kehat et al. 2001). This protocol was thereafter utilized as the standard method to generate CMs from hESCs, but is flawed by several limitations. These include the low yield of CMs, which are as low as less than 1 % of the total cell population obtained by spontaneous hESC differentiation. Moreover, stimulating cell growth with FBS may not be the most suitable strategy for CBT because of the potential risks of contamination associated with serum. Innovative enrichment, purification and selection protocols have thus been established to drive cardiac differentiation toward relatively pure homogeneity. Defined culture media have been developed to improve the efficiency of CM derivation from hESCs. For instance, earlier studies demonstrated that co-culturing hESCs with END2, a mouse visceral endoderm-like cell-line, increased the percentage of contracting EBs to 35 % after 12 days in culture (Mummery et al. 2003). Subsequent studies showed that the cardiac inductive activity of END2 may be mimicked by culturing hESCs in insulin-free, serum-free medium supplemented with high concentrations of prostaglandin I2 (PGI2) (Xu et al. 2008a). Alternatively, specific growth factors involved in the initiation of the signalling pathways that control cardiogenesis can be used to favour CM generation from hESCs. For instance, activin 4 and BMP4, both members of the transforming growth factor β (TGFβ) family, may sequentially be added to the differentiation medium to favour efficient cardiac development. A monolayer of undifferentiated hESCs is plated on Matrigel before being exposed to activin A for 1 day and then to BMP5 for further 4 days, followed by removal of these growth factors and replacement with a serum-free medium for about 3 weeks. This protocol yields 30 % of CMs as compared to the conventional serum-induced differentiation (Laflamme et al. 2007). Alternatively, cardiac induction may be initiated by stimulating the Wnt/β-catenin signalling pathway, which controls cardiogenesis in zebrafish, *Xenopus* and mouse, with Wnt3 for 2 days in serum-containing medium, while activin A is added during the early phases of differentiation along with Wnt3 to accelerate CM formation. The final CM yield of this treatment is thus enhanced to ~50 % (Paige et al. 2010). Consistently, dickkopf homolog 1 (DKK1) adverses CM differentiation when is supplemented during the early stages of the protocol by inhibiting Wnt signalling (Tran et al. 2009). Nevertheless, CM generation is favoured when DKK1 is added at later stages (5–11 days) (Paige et al. 2010).

Finally, a staged protocol has been devised to recapitulate the microenvironment of developing mouse embryo by applying five growth factors at different time points during the differentiation process. Human ESCs are first stimulated with BMP4 for 1 day to form EBs and then challenged with BMP4, activin A, and basic fibroblast growth factor (bFGF) from days 1 to 4 to induce mesoderm. The development of cardiovascular lineages is promoted by the subsequent addition of vascular endothelial growth factor (VEGF), DKK1 and bFGF. The culture is maintained in hypoxic environment for the first 10–12 days and the final CM yield is ~40 % (Yang et al. 2008; Kattman et al. 2011).

The efficiency of CM differentiation might also be improved by targeting the signalling pathways involved in cardiogenesis by exploiting selective small molecule inhibitors or activators. For instance, low concentrations (10 μM) of SB203580, a specific inhibitor of p38 mitogen-activated protein kinase (MAPK), enhances CM formation from hESCs maintained in END2-conditioned medium by ~2 % (Graichen et al. 2008); the same effect is obtained when SB203580 is added to serum-free, insulin-free medium supplemented with high doses of PGI_2 (Xu et al. 2008a). Moreover, CM differentiation may be stimulated by treating hESCs with BMP2, another cardiogenic inducer, and SU5402, a FGF receptor blocker, albeit this effect is specific to well defined cell lines and culture conditions (Burridge et al. 2007; Yang et al. 2008). Finally, the demethylating agent 5-azacytidine, ascorbic acid, and cyclosporine A, but not retinoic acid and DMSO, were all reported to exert efficient cardiogenic effects (Passier et al. 2006; Xu et al. 2012).

The protocols illustrated above were conceived to direct cardiac differentiation from pluripotent hESCs; however, while the efficiency of CM differentiation is improved by such treatments, hESCs-derived cultures still contain cell types deriving from all three germ layers that might reduce the therapeutic outcome of CBT or even exert a pro-tumorigenic effect (Xu et al. 2012; Bernstein 2012). Several methods have been developed to enrich CMs from hESCs-derived cultures and enhance the purity of cell populations to be utilized for clinical applications. Manual dissection and dissociation of contracting areas from human ES cells-derived EBs could produce up to 70 % cells positively stained for cardiac markers (Mummery et al. 2003). A less labour intensive method is based on discontinuous Percoll gradient followed by centrifugation: most of CMs accumulate in the high percentage Percoll layer, thereby leading to three to sevenfold enrichment up to 70 % (Xu et al. 2002) and, eventually, 95 % CMs when differentiation is induced with activin A and BMP4 (Laflamme et al. 2007). Importantly, Percoll enriched CMs have successfully been employed in cardiac regeneration studies with no evidence of teratoma formation (Xu et al. 2012). A third strategy takes advantage from the high mitochondrial content of CMs as compared to non-cardiac cells. The fluorescent dye tetramethylrhodamine methyl ester perchlorate (TMRM) reversibly labels mitochondria and does not impair cell vitality. Cell sorting of differentiating human EBs carried out by exploiting TMRM fluorescence leads to a CM purity >90 %. When these cells were injected in immunodeficient mouse hearts, they showed a remarkable long-term (8 weeks) survival and did not form teratomas (Hattori et al. 2010). These enrichment protocols, although useful to obtain high purity CM populations from

human EBs, present several limitations that could affect their therapeutic translation. None of them permit to obtain cardiac progenitor cells (CPCs) that could be useful for the reconstruction of injured myocardium in virtue of their ability to differentiate into multiple cells types, such as CMs, smooth muscle cells and endothelial cells. Moreover, mechanical dissection can only be performed towards the end of the differentiation process, when a sufficient number of contracting foci is detectable. Percoll gradient is also less efficient when is conducted at the beginning of human ES cell differentiation. Finally, TMRM purification is recommendable at the late stage of differentiation (from day 20 on), as at earlier stages many non-cardiac cells may be selected by this approach (Dubois et al. 2011; Elliott et al. 2011). Lineage-specific surface antigens have, therefore, been identified to selectively enrich CMs through fluorescence-activated or magnetic-assisted cell separation (FACS and MACS, respectively) procedures. Two cell surface cardiac markers have recently been associated to hESCs-derived CMs: vascular cell adhesion molecule 1 (VCAM1) and signal-regulatory protein alpha (SIRPA). FACS-based selection for $SIRPA^+$ cells leads to a population of 90–98 % cardiac Troponin T^+ ($cTnT^+$) cells (Dubois et al. 2011), while MACS-based sorting of $VCAM1^+$ cells results in a yield of 95 % $cTnT^+$ cells (Uosaki et al. 2011). Likewise, activated leukocyte cell adhesion molecule (ALCAM) is expressed in hESCs-derived CMs and is amenable to MACS. This approach leads to 58 % of the enriched population positive for both ALCAM and the cardiac marker α-MHC (Rust et al. 2009). These strategies present a number of hurdles as well. While FACS has a low throughput but may target multiple markers on individual cells, MACS displays a higher throughput but is limited to a single marker. Moreover, the percentage of hESCs-derived CMs expressing cTnT, SIRPA and VCAM1 amounts to only 37 % (Elliott et al. 2011), while it is still unknown whether ALCAM co-localizes with SIRPA and/or VCAM1 or identifies an additional CM population (Xu et al. 2012). It has, therefore, recently suggested to targeting non-cardiac markers to deplete non-cardiac cells and obtain pure CM-enriched cultures. Cell antigens exploited for this purpose include β-type platelet-derived growth factor receptor (PDGFRB; to exclude smooth muscle cells), platelet endothelial cell adhesion molecule-1 (PECAM-1; to exclude endothelial cells), and THY (to exclude fibroblasts) (Dubois et al. 2011).

Genetic selection provides the opportunity to purify hESCs-derived cardiomyocytes by placing a reporter gene or selectable marker under the control of a cardiac-specific promoter. This strategy, initially developed to obtain cultures consisting of >99.6 % CMs from mESCs (Klug et al. 1996), associated to the paucity of cell surface antigens has led to the development of several reporter hESC lines. Genetically-modified cells are thus induced to go through the differentiation process and, subsequently, differentiated CMs can be sorted by FACS, if the transgene encodes for a fluorescent protein (i.e. green fluorescent protein and mCherry), or selected by antibiotics (i.e. neomycin or geneticin), if the transgene determines antibiotic resistance (Wong and Bernstein 2010; Xu et al. 2012). Cardiac promoters that have been exploited to obtain purified CM culture include mouse or human cardiac α-MHC and human myosin light chain 2v (MLC2V). This strategy may yield up to 99 % of pure CMs that exhibit human adult cardiac genes and fire action potential

similar to those recorded in fetal CMs (Kita-Matsuo et al. 2009; Wong and Bernstein 2010). Moreover, CMs generated through FACS or antibiotic resistance do not lead to teratoma formation (Xu et al. 2008a) and generate contractile forces similar to those of rat neonatal CMs (Kita-Matsuo et al. 2009). Nevertheless, their regenerative impact in the ischemic or failing heart remains to be elucidated; moreover, developing stable expression lines is an expensive and time consuming process that could be hampered by random integration of the selected transgene in the host genome, thereby causing genetic modifications of stem cells lines. This hurdle can be overcome by adopting the so-called dual fluorescence resonance energy transfer 'molecular beacon' (MB) technology for transient, real-time detection of gene expression during hESC differentiation. Molecular beacons are single-stranded oligonucleotide nanoscale probes with a fluorophore at the 5′ end and a quencher at the 3′ end. In the absence of their complementary target, MBs adopt a hairpin structure that causes the fluorophore to come in close contact with the quencher, thereby reducing the overall fluorescence. However, hybridization with a homologous sequence target opens the hairpin and separated the two opposite ends, thus leading to an increase in fluorescence. This strategy has been modified to detect the expression of specific cardiac mRNAs (i.e. Oct4 and MHC1) by using dual fluorescence resonance energy transfer (FRET) associated with FACS (King et al. 2011; Ban et al. 2013). Recently, MHC1-MBs^{+} CMs have been shown to engraft up to 4 weeks in an AMI model without forming teratomas and significantly improving LVEF (by ~10 %) (Ban et al. 2013).

An extensive molecular characterization has demonstrated that hESCs-derived CMs express: (1) cardiac transcription factors, such as GATA-4, MEF-2, and Nkx2.5; (2) sarcomeric and myofibril proteins, such as α- and β-MHC, atrial and ventricular forms of MLC (MLC2A and MLC2V), tropomyosin, cTnT, cardiac Troponin I, α-actinin and desmin; and (3) metabolic regulators of cardiac development, such as ANF, myoglobin and creatine kinase-MB (Kehat et al. 2001; Xu et al. 2002). Nevertheless, hESCs-derived CMs present cytological and ultrastructural organization typical of foetal/neonatal, rather than adult, CMs: they are mononuclear cells, featured by a round, tri- or multi-angular shaped morphology, and with a high nuclear:cytoplasmic ratio. They, however, adopt a more elongated structure during maturation which is coupled to a progressive ultrastructural development from an irregular myofibrillar pattern to the generation of well-identifiable sarcomers at the end of the differentiation process. Accordingly, at this stage, some sarcomers show recognizable A-, I-, and Z-bands, albeit hESCs-derived CMs always lack T tubules (Snir et al. 2003; Passier et al. 2008). Likewise, hESCs-derived CMs display immature electrophysiological features. The beating rate in spontaneously contracting hEBs ranges between 30 and 130 bpm and undergoes positive and negative chronotropic regulation in response to adrenergic and cholinergic agonists, respectively. This stable pacemaker activity within hEBs is associated to functional gap junctions, comprising connexins 43 and 45, which enable the synchronized spreading of the electrophysiological signal through the cell aggregate. It must, however, be pointed out that: (1) connexin 45 expression is restricted at the early stages of in vivo development and (2) the beating activity may cease after the first

9 weeks of culture (Blazeski et al. 2012). Upon differentiation, hESCs-derived CMs exhibit a mixture of electrophysiological profiles that resemble those of the immature adult heart and are classified as nodal-, atrial-, and ventricular-like. This "functional signature" may vary depending on hESC lines, differentiation protocols and time of differentiation, as reviewed by Blazeski et al. (2012), and displays heterogeneous properties even among cell outgrowths deriving from the same EB. Consistent with the foetal/embryonal phenotype of the action potentials they trigger, hESCs-derived atrial- and ventricular-CMs are more depolarized (−40/−50 mV) than their adult counterparts (Passier et al. 2008; Blazeski et al. 2012). Moreover, the majority of hESCs-derived CMs shows a high degree of automaticity and spontaneously fires in absence of external stimuli; quiescent cells, however, trigger single action potentials upon stimulation and are, therefore, functionally active. The up-stroke is shaped by voltage-gated Na^+ channels (mainly encoded by $Na_V1.5$), but is significantly slower in ventricular hESCs-derived CMs than in adult cells (12 *vs.* 118 V/s). Ca^{2+} entry through L-type voltage-gated Ca^{2+} channels (encoded by $Ca_V1.2$) maintains the depolarization, while repolarization is accomplished by transient outward K^+ channels (I_{to}, encoded by Kv4.3), slow delayed rectifier K^+ channels (I_{Ks}, encoded by Kv7.1), and inward rectifier K^+ channels (I_{Kr}, encoded by Kv11.1 or human ether-a gò-gò related gene 1—HERG1). The spontaneous firing is initiated by voltage gated Na^+ channels with the contribution of the pacemaker current I_f (or funny current, mediated by hyperpolarization-activated cyclic nucleotide-gated channel 1 or HCN1). In general, most of these ion channels undergo a remarkable up-regulation over a long maturation period (i.e. 3–8 months) which moves the action potential shape toward a more adult phenotype, but I_f and HCN1 are down-regulated; this process is significantly slower as compared to the differentiation of mESCs-derived CMs which acquire an adult electrophysiological phenotype in approximately 3 weeks (Sartiani et al. 2007; Passier et al. 2008; Blazeski et al. 2012; Robertson et al. 2013). The mechanism of excitation-contraction coupling in hESCs-derived CMs is still highly debated. Overall, their intracellular Ca^{2+} signalling machinery is more immature as related to adult CMs, which show a positive force-frequency relationship due to the concerted interaction between voltage-operated Ca^{2+} inflow and RyRs activation at the dyadic junctions. Conversely, hESCs-derived CMs display a negative force-frequency, which reflects the lack of T-tubules and the immaturity of Ca^{2+} cycling across sarcoplasmic reticulum (SR) membranes. Furthermore, intracellular Ca^{2+} mobilization in these cells is dampened by a number of limiting factors, including: (1) low SR Ca^{2+} content, SERCA2A and RyR2 expression, which render them less sensitive to electrical stimulation and caffeine application; (2) absence of the SR regulatory proteins calsequestrin, junctin and triadin, which control SR Ca^{2+} content and RyR2 activation; (3) U-shaped propagation of electrically-induced intracellular Ca^{2+} waves. All of these features are typical of foetal/neonatal CMs and concur with the scarce contribution of RyRs2-induced Ca^{2+} release to the activation of the contractile machinery (Kong et al. 2010; Blazeski et al. 2012; Robertson et al. 2013). Consistently, abating RyRs2 signalling reduces the beating rate, but does not suppress contraction development. This immature phenotype is further supported by the observation that $InsP_3R2$ may be involved in intracellular Ca^{2+} mobilization and control of contraction rate (Jaconi et al. 2000; Robertson et al. 2013).

2.4 Advantages and Disadvantages of Embryonic Stem Cells as Source for Cardiac Regeneration

Among the different stem cell sources that can potentially be used to promote cardiac regeneration, hESCs stand out as the most promising candidates in virtue of their ability to differentiate into genuine CMs. Pioneering preclinical studies demonstrated that mESCs and mESCs-derived CMs may engraft and regenerate infracted myocardium by improving its contractile function (Christoforou and Gearhart 2007). Initially, hESCs-derived CMs were shown to restore the pacemaking activity in the heart of swines and guinea pigs with complete atrio-ventricular block or cryoablation, respectively (Passier et al. 2008). Later, it was demonstrated that hESCs-derived CMs survive, proliferate and mature for at least 12 weeks after injection into the healthy myocardium of immunodeficient mice or rats. However, grafted cells formed a syncytium that is separated from host tissue by a fibrotic patch secreted both by resident and transplanted CMs (Laflamme et al. 2005; Dai et al. 2007; Passier et al. 2008). Unfortunately, the engraftment success rate dropped from 90 to 18 % when hESCs-derived CMs were infused into a murine model of AMI. The same study disclosed that a pro-survival cocktail, containing components of the extracellular matrix and anti-death factors, improved graft survival and, consequently, ventricular contractility up to 4 weeks post-transplantation (Laflamme et al. 2005). Additional studies confirmed that hESCs-derived CMs represent a feasible tool to induce cardiac repair in rodent models of AMI (van Laake et al. 2007a, b, 2008; Leor et al. 2007; Caspi et al. 2007a). Nevertheless, no clinical trials have been launched yet as a consequence of the major challenges that remain to be overcome before the clinical translation of hESCs-derived CMs. First, the use of human-derived cells has raised serious ethical and legal concerns and it does not easily gain the appreciation of the public opinion (Joggerst and Hatzopoulos 2009; Wong and Bernstein 2010; Jakob and Landmesser 2013; Pfister et al. 2014). Second, functional experiments unveiled that their effect on cardiac performance is only transient. For instance, even when the dose of hESCs-derived CM injected into injured heart is tripled, no functional improvement is observed after 12 weeks (van Laake et al. 2007a, b, 2008). This therapeutically relevant hurdle might be solved by using hESCs-derived cardiac progenitor cells (CPCs), which possess a superior ability to integrate within the injured heart and restore cardiac vascularisation (Wong and Bernstein 2010; Bernstein 2012). However, the transient improvement of ventricular function induced by hESCs-derived CMs may also reflect the mismatch between the beating rates of human *vs.* rodent CMs (60–100 *vs.* 300–600 bpm). This would reflect in the functional uncoupling that leads to the failure of long-term treatment (Passier et al. 2008). Therefore, rodent hearts might not provide the most suitable model to study the regenerative potential of hESCs, and larger animals, such as pigs and primates, with beating rates comparable to those of humans could be used to address this issue. Third, hESCs may give raise to teratocarcinomas at the implantation site. Albeit these teratomas are regarded as benign in vivo, some studies reported the expression of malignant markers in some cells. The injection of hESCs-derived

CMs is predicted to alleviate this side effect, but these findings raise ethical concerns about the safety of their clinical use. Fourth, allogenic hESCs are obtained from embryos and obviously do not retain the same genome as the patients. It turns out that, although they are immunologically immature, hESCs-based therapy requires an immunosuppressive regimen (i.e. tacrolimus or cyclosporine) to prevent immune rejection after transplantation. Induced pluripotent stem (iPS) cells may be generated by reprogramming adult autologous differentiated cells (Takahashi and Yamanaka 2006; Takahashi et al. 2007) and then directed to differentiate towards CMs. Such strategy circumvents the limitation imposed by ethical concerns and immunological reaction, but is extremely expensive, time consuming and not exempt by risk (teratoma formation in vivo, virus as vectors, genomic rearrangements and epigenetic mutations) (Bernstein 2012; Akhmedov and Marín-García 2013). However, protocols for cardiac differentiation of hESCs have been significantly improved to attenuate the risk of teratoma formation, favour stem cell engraftment, and increase functional improvement (Mummery et al. 2012; Bernstein 2012). For instance, alternative culture products and feeder-free culture systems have been devised to obtain and maintain hESCs under animal- and xenobiotic-free conditions (Bernstein 2012). Moreover, earlier hESC lines have been discarded for clinical use as long-term cultures lead to chromosomic and genomic instabilities; therefore, new hESC lines are required that should be maintained under conditions that do not compromise genomic integrity improvement (Mummery et al. 2012; Bernstein 2012). This goal could, for instance, be achieved by exploiting culture media that induce adult-like energy metabolism (Maher and Xu 2013).

2.5 Future Trends in the Therapeutic Application of hESCs for Cardiac Regeneration

In the next future, unveiling the molecular determinants of cardiogenesis will favour the development of more robust and reliable methodologies to promote hESCs differentiation into cardiac myocytes. Recent work clearly showed that miR-1 and miR-133 are enriched in mESCs and hESCs-derived CMs and are involved in the acquisition of a cardiac phenotype. Intriguingly, miR-1 over-expression increases Nkx2-5 expression in hESCs and results in more than a threefold higher number of beating hEBs as compared with wild-type cells (Ivey et al. 2008). Epigenetic manipulation could provide an additional means to control stem cell fate. The histone deacetylase inhibitor trichostatin A (TSA) may be administrated to mESCs to favor cardiogenesis by enhancing the expression of Nkx2-5, ANF, and β-MHC. Moreover, mESCs may be committed toward the cardiac lineage by the ectopic expression of Baf60c, a cardiac-enriched subunit of the Swi/Snf-like BAF chromatin remodeling complex, and the cardiac transcription factors GATA4 and Tbx5 (Takeuchi and Bruneau 2009). These observations remain to be confirmed in hESCs, but pave the way to design alternative protocols to promote cardiac differentiation for regenerative purposes. In this context, it will be important to understand how specifically directing the differentiation process towards the nodal or working (atrial

and ventricular) phenotype. Currently available protocols do not distinguish between these two different cardiac subtypes which might induce undesired off-target effects in recipient hearts. While working-type cardiomyocytes are more suitable for cardiac regeneration, enriched nodal-type cardiomyocytes are more amenable for restoring the pacemaker function in arrhythmic patients. Contamination with nodal-type cells might thus enhance the risk for arrhythmia in ischemic/failing patients and represents a potential risk for hESCs-based therapy of CD (Maher and Xu 2013). Profiling the panel of cell surface antigens that identify cardiac progenitor pools and improving non-genetic selection strategies will permit to enrich the hESCs-derived population of CMs ready for clinical use. Recent advances in tissue engineering are predicted to enhance long-term graft and survival of hESCs-derived CMs. A number of 3D human cardiac patches have been constructed that contain embryonic fibroblasts, human endothelial cells, and hESCs-derived CMs (Caspi et al. 2007b; Lesman et al. 2010). This multicellular preparation is fully vascularized and capable of producing spontaneous and synchronized contractions that are propagated by gap junction connecting adjoining CMs (Caspi et al. 2007b). Relevant to reparative purposes, such tissue-engineered human vascularized cardiac muscle forms stable biografts in the heart of recipient rats (Lesman et al. 2010). It will be important to develop a reliable and reproducible strategy to prevent immune rejection of injected cells. Preliminary studies conducted on patients suffering from spinal cord injury and receiving hESCs-derived oligodendrocytes did not show any deleterious consequence after 6 months of moderate immunosuppression. The lower drug doses employed, as compared to whole organ transplantation, are justified by the lack of antigen presenting cells within the cellular graft, which reduces its immunogenicity (Menasché 2012). Finally, hESCs might contribute to cardiac regeneration by means other than cell replacement, i.e. by paracrine release of cardioprotective mediators. The injection of hESCs-derived CMs in a mouse model of AMI has been shown to increase angiogenesis, reduce apoptosis, infarct scar and myocardial fibrosis, and improve LVEF even in the absence of long-term engraftment. This observation suggests that hESCs-derived CMs secrete soluble factors with the potential to stimulate the endogenous mechanisms of cardiac healing. The identification and isolation of such biofactors could lead to the synthesis of "off-the-shelf" drugs to restore myocardial structure and function with no need of CBT and circumventing all its associated hurdles and concerns.

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