

# Inducing Embryonic Stem Cells to Become Cardiomyocytes

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**Abstract** Many forms of heart disease are associated with a decrease in the number of functional cardiomyocytes. These include congenital defects (e.g. hypoplastic and noncompaction syndromes) as well as acquired injuries (e.g. exposure to cardiotoxic agents or injuries resulting from coronary artery disease, hypertension, or surgical interventions). Although the adult mammalian heart retains some capacity for cardiomyocyte renewal (resulting from cardiomyocyte proliferation and/or cardiomyogenic stem cell activity), the magnitude of this regenerative process is insufficient to effect repair of substantively damaged hearts. It has become clear that exogenous cardiomyocytes transplanted into adult hearts are able to structurally and functionally integrate. It has also become clear that embryonic stem cells (ESCs), as well as induced progenitors with ESC-like characteristics, are able to generate bona fide cardiomyocytes in vitro and in vivo. These cells thus constitute a potential source of donor cardiomyocytes for therapeutic interventions in damaged hearts. This chapter reviews spontaneous cardiomyogenic differentiation in ESCs, methods used to generate enriched populations of ESC-derived cardiomyocytes, and current results obtained after engraftment of ESC-derived cardiomyocytes or cardiomyogenic precursors.

**Keywords** Cardiac differentiation • Intracardiac engraftment • Cell therapy

## 1 Introduction

The structure and cellular composition of the adult mammalian heart are complex; consequently, myocardial disease can manifest itself at many different levels, and can impact multiple structures and cell types (valves, coronary arteries, capillaries,

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endothelial cells, veins, interstitial fibroblasts, nodal cells, conduction system cells, working cardiomyocytes, etc.). Advances in surgical and pharmacologic interventions, as well as the development of electrophysiologic and mechanical devices, have steadily advanced and currently provide a wide variety of viable treatments for many forms of heart disease. The elucidation of the molecular underpinnings of cell lineage commitment and morphogenesis provide additional avenues of treatment, particularly in the area of angiogenesis. Unfortunately, the ability to promote widespread replacement of lost contractile units (i.e. cardiomyocyte replacement) has remained elusive.

Developmental and molecular studies have identified progenitor cells which give rise to cardiomyocytes in the developing heart. Proliferation of immature but contracting cardiomyocytes is a major contributor to the increase in cardiac mass observed during fetal development. The proliferative capacity of cardiomyocytes decreases markedly in postnatal life. Additionally, several progenitor cell populations with cardiomyogenic activity identified during development are depleted or have lost their ability to form new cardiomyocytes in neonatal life. Nonetheless, evidence for cardiomyocyte proliferation and/or apparent cardiomyogenic stem cell activity has been reported in the adult heart. For example, quantitation of radioisotope incorporation into cardiomyocyte nuclei of individuals alive during atmospheric atomic bomb detonations suggested an annual cardiomyocyte renewal rate of approximately 1% in young adults [1], a value remarkably similar to that extrapolated from shorter pulse/chase tritiated thymidine incorporation studies in mice [2]. Although the findings of these studies collectively are more consistent with the notion of cardiomyocyte renewal via proliferation, they do not rule out potential contributions from cardiomyogenic stem cells. Indeed, studies employing an elegant conditional reporter transgene system suggested stem-cell-based regeneration following injury in adult mice [3]. The notion of cardiomyocyte renewal in the adult heart has been with us for a long time – at issue is the magnitude of the regenerative response, a point which is the subject of intense research and debate among cardiomyocyte aficionados. What is clear is that the adult heart lacks the ability to reverse damage following the loss of large numbers of cardiomyocytes.

Studies from the 1990s demonstrated that donor cells could successfully engraft the hearts of recipient animals. Proof-of-concept experiments showed that cardiomyocytes from enzymatically dispersed fetal mouse hearts structurally integrated into the hearts of adult recipients following direct intracardiac injection [4, 5]. Subsequent analyses demonstrated that the donor cardiomyocytes formed a functional syncytium with the host myocardium, using the presence of intracellular calcium transients as a surrogate marker for contractile activity [6]. Although promising, it was soon apparent that only a small fraction of the injected cardiomyocytes survived and engrafted [7], a problem which remains a major obstacle for clinical efficacy of this approach. Nonetheless, several studies have reported that intracardiac injection of fetal cardiomyocytes could preserve cardiac function following experimental injury in rodents [8–10].

In light of these observations, considerable effort has been invested to identify potential sources of donor cardiomyocytes, or alternatively progenitor cells with

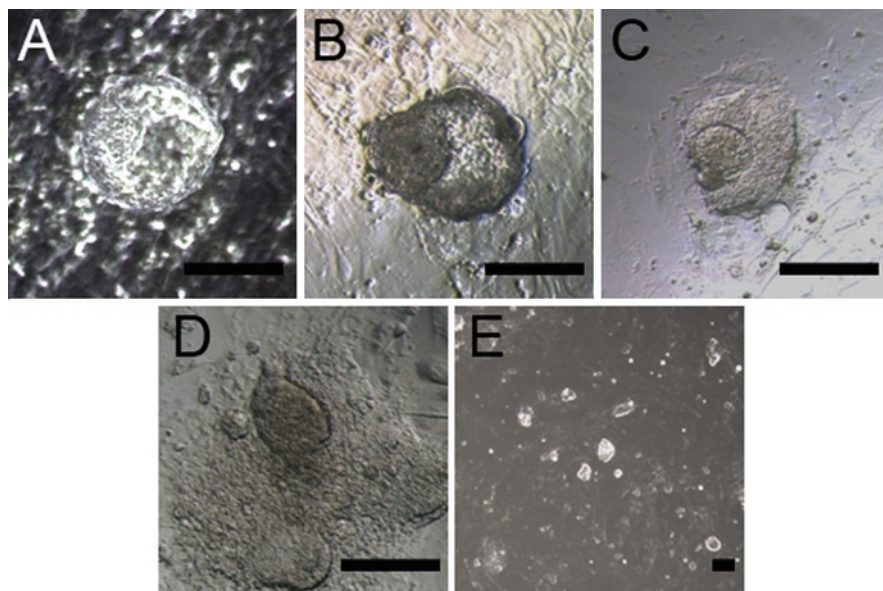
cardiomyogenic activity. Toward that end, many cells with apparent cardiomyogenic activity have been reported in the recent literature, a remarkable observation given that the intrinsic regenerative rate in the adult myocardium is quite low. Many factors likely contribute to this phenomenon. For example, the presence of multiple markers, or alternatively the transient expression of different markers, could result in an individual cell or cell lineage being categorized as multiple cells/lineages. The ability of some cell types to fuse with cardiomyocytes [11] could result in their false identification as cardiomyogenic stem cells. The relative rigor of the assays employed to detect cardiomyogenic activity could also contribute to the identification of false positives. It is also possible that reprogramming during *in vitro* propagation unmasked or enhanced cardiomyogenic potential. Given the intense activity in the field, it is likely that the true *in vitro* and *in vivo* cardiomyogenic activity of the various progenitor cells identified to date will rapidly be either validated or repudiated.

It is well established that embryonic stem cells (ESCs) are able to generate bona fide cardiomyocytes [12]. ESCs are derived from the inner cell mass (ICM) of preimplantation embryos [13, 14]. ESCs can be propagated *in vitro* in an undifferentiated state, and when allowed to differentiate can form endodermal, ectodermal, and mesodermal derivatives *in vitro* and *in vivo*. ESCs thus constitute a potential source of donor cardiomyocytes (or alternatively, donor cardiomyogenic progenitors) for therapeutic interventions targeting diseased hearts. In this chapter we review the spontaneous cardiomyogenic differentiation in ESCs, the various methods which have been developed to generate enriched populations of ESC-derived cardiomyocytes, and the current status of preclinical studies aimed at regenerating myocardial tissue via engraftment of ESC-derived cardiomyocytes or cardiomyogenic precursors. We then consider the challenges which must be overcome for successful translation to the clinic.

## 2 ESCs and Spontaneous Cardiomyogenic Differentiation

After fertilization, initial growth of the preimplantation mammalian embryo is characterized by rapid cell division. Cells within the embryo begin to differentiate at the 16-cell stage (morula). As development proceeds, cells on the periphery of the morula give rise to trophoblasts (which, together with maternal endometrium, form the placenta) and cells in the center of the morula give rise to the ICM (which forms the embryo). The resulting blastocyst remains surrounded by the zona pellucida. Blastocysts can be cultured on feeder layers of mitomycin-treated mouse embryonic fibroblasts (MEFs). In the example shown in Fig. 1a, the MEFs were derived from transgenic mice carrying a transgene encoding leukemia inhibitory factor (LIF). LIF activates the Janus kinase/signal transducer and activator of transcription (JAK/STAT) and mitogen-activated protein kinase pathways and suppresses differentiation in mouse ESCs (but is not required for generating human ESCs).

After several days of culture, the zona pellucida of the preimplantation embryo will rupture, allowing the outgrowth of both trophoblasts and ICM cells (Fig. 1b–d). The two cell types were readily distinguished by phase-contrast microscopy, with the

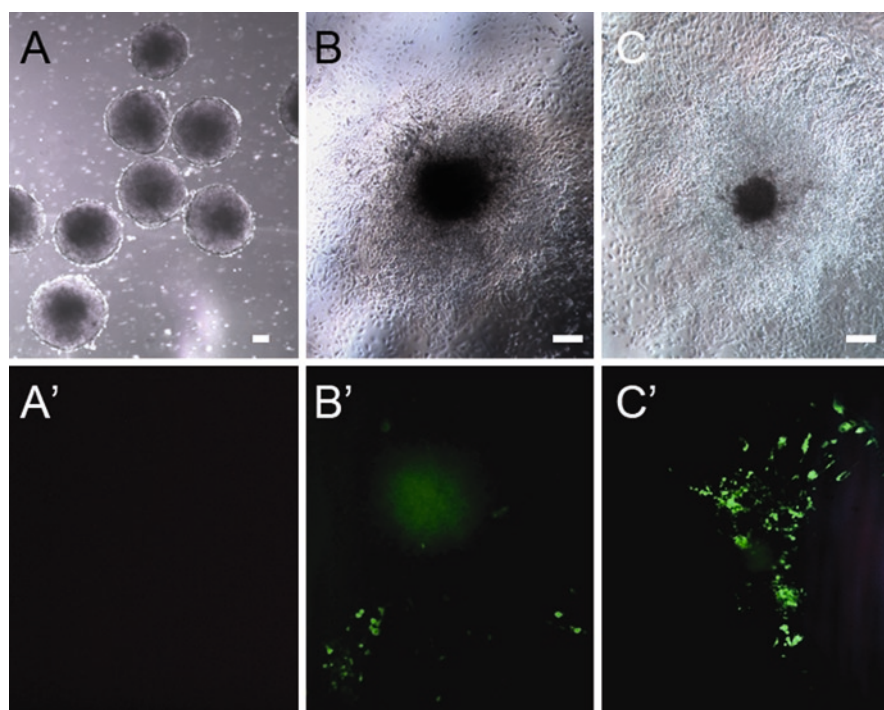


**Fig. 1** Derivation of mouse embryonic stem cell (ESC) lines. **(a)** Blastocyst isolated at 3.5 days post coitus and culture for 3 days on a mouse embryonic fibroblast (MEF) feeder layer. Note the presence of the zona pellucida (a refractile ring surrounding the embryo) and the inner cell mass. **(b–d)** Blastocysts after 6, 7, and 8 days of culture on the MEF feeder layer. Note that the zona pellucida ruptures with time, releasing inner cell mass and trophoblast cells. **(e)** ESC lines after three passages. Bar 100  $\mu\text{m}$

ICM derivatives exhibiting a very dense, refractile morphology. Clusters of refractile cells were then physically isolated, dispersed, and replated onto MEF feeder layers. This process was repeated until clonal ESC lines were established (Fig. 1e). Mouse ESC lines can be propagated extensively in an undifferentiated state as long as care is exercised to maintain high levels of LIF and to limit colony size.

Early studies demonstrated that, when cultivated in suspension, ESCs form multicellular aggregates which have been termed “embryoid bodies” (EBs) [14]. Stochastic signaling between different cell types within the EBs mimics *in vivo* developmental induction cues, and upon further differentiation (either in suspension or adherent culture) the EBs give rise to ecto-, endo-, and mesodermal derivatives. Wobus and colleagues [15] developed a very useful technique to generate EBs with reproducible ESC content (which in turn resulted in more reproducible patterns of differentiation). This entailed placing microdrops of medium seeded with a fixed number of undifferentiated ESCs on the inner surface of a tissue culture dish lid. The lid was then gently inverted so as to prevent mixing of the microdrops, and was placed on a tissue culture dish containing medium. The resulting “hanging drops” provide an ideal environment for the ESCs to coalesce and form EBs in a highly reproducible manner. Subsequent studies by Zweigerdt and colleagues demonstrated that EBs with reproducible ESC content could be generated in bulk in tissue culture dishes on rotating devices [16] or in stirred bioreactors [17].

To document cardiomyogenic differentiation using the hanging drop approach, ESCs were generated using blastocysts derived from myosin heavy chain (MHC)-enhanced green fluorescent protein (EGFP) transgenic mice. These mice carry a transgene comprising the cardiomyocyte-restricted  $\alpha$ -MHC promoter and an EGFP reporter. The transgene targets EGFP expression in cardiomyocytes [6], and thus provides a convenient reporter to trace cardiomyogenic activity in differentiating ESC cultures, as illustrated in Fig. 2 (Fig. 2a–c shows phase-contrast images of the EBs and adherent cultures, and Fig. 2d–f shows epifluorescence images of the same field). Individual dispersed ESCs were plated in hanging drops; after several days in culture, the ESCs formed EBs which continued to grow and differentiate. No EGFP epifluorescence was apparent, consistent with the absence of cardiomyogenic differentiation at this stage (Fig. 2a). The EBs were transferred from



**Fig. 2** Timeline of cardiomyogenic differentiation in mouse ESCs carrying a myosin heavy chain (MHC)-enhanced green fluorescent protein (EGFP) reporter transgene. (**a**, **a'**) Phase-contrast and epifluorescence images, respectively, of embryoid bodies (EBs) generated by the hanging drop procedure after 4 days of suspension culture. The absence of EGFP epifluorescence indicates that cardiomyocyte differentiation has not yet occurred. (**b**, **b'**) Phase-contrast and epifluorescence images, respectively, of an EB after 5 days of suspension culture and 2 days of adherent culture. A few scattered cells with EGFP epifluorescence indicates the initial onset of cardiomyocyte differentiation. (**c**, **c'**) Phase-contrast and epifluorescence images, respectively, of an EB after 5 days of suspension culture and 5 days of adherent culture. Most cardiomyogenic differentiation has occurred by this time. Bar 100  $\mu$ m

suspension culture to adherent culture after 5 days of differentiation. Expression of the cardiomyocyte-restricted reporter transgene was first detected after 7 days of differentiation (i.e. 5 days of suspension culture and 2 days of adherent culture; Fig. 2b); however, contractile activity was not apparent until 3 days of differentiation. This reflected the time differential between the induction of myofiber structural protein gene expression (and, consequently, activation of the reporter transgene expression) and the assembly of functional myofibers and the requisite intracellular machinery for the generation and propagation of action potentials and calcium transients. It was also apparent that cardiomyocytes constituted only a small fraction of the total cell population during spontaneous ESC differentiation (Fig. 2c). With the development of human ESC lines [18], in vitro cardiomyocyte differentiation was rapidly observed and characterized [19].

### 3 Inducing ESCs to Produce Cardiomyocytes

Numerous approaches have been developed to generate enriched cultures of ESC-derived cardiomyocytes (Table 1). Perhaps the most obvious approach entails the identification of growth factors which enhance cardiomyocyte differentiation. Indeed,

**Table 1** Approaches to enhance cardiomyocyte yield from embryonic stem cells (ESCs)

Approach	Comments	References
Growth factors	Retinoic acid enhanced cardiomyocyte differentiation in mouse ESCs	[20]
	Exogenous glucose, amino acids, vitamins, and selenium enhanced cardiomyocyte differentiation in mouse ESCs	[21]
	LIF enhances and inhibits cardiomyocyte commitment and proliferation in mouse ESCs in a developmental stage-dependent manner	[22]
	Reactive oxygen species enhanced cardiomyocyte differentiation in mouse ESCs	[23, 24, 25, 26]
	Endoderm enhanced cardiomyocyte differentiation in mouse ESCs	[27, 28]
	A TGF/BMP paracrine pathway enhanced cardiomyocyte differentiation in mouse ESCs	[29]
	Activation of the MEK/ERK pathway enhanced cardiomyocyte differentiation in mouse ESCs	[30]
	Verapamil and cyclosporine enhanced cardiomyocyte differentiation in mouse ESCs	[31]
	5-Aza-2'-deoxycytidine enhanced cardiomyocyte differentiation in human ESCs	[32]
	Endoderm cell lines enhanced cardiomyocyte differentiation in human ESCs	[33, 34]
	Ascorbic acid enhanced cardiomyocyte differentiation in human ESCs	[35]
	Directed differentiation with activin A and BMP4 in monolayers of human ESC	[36]

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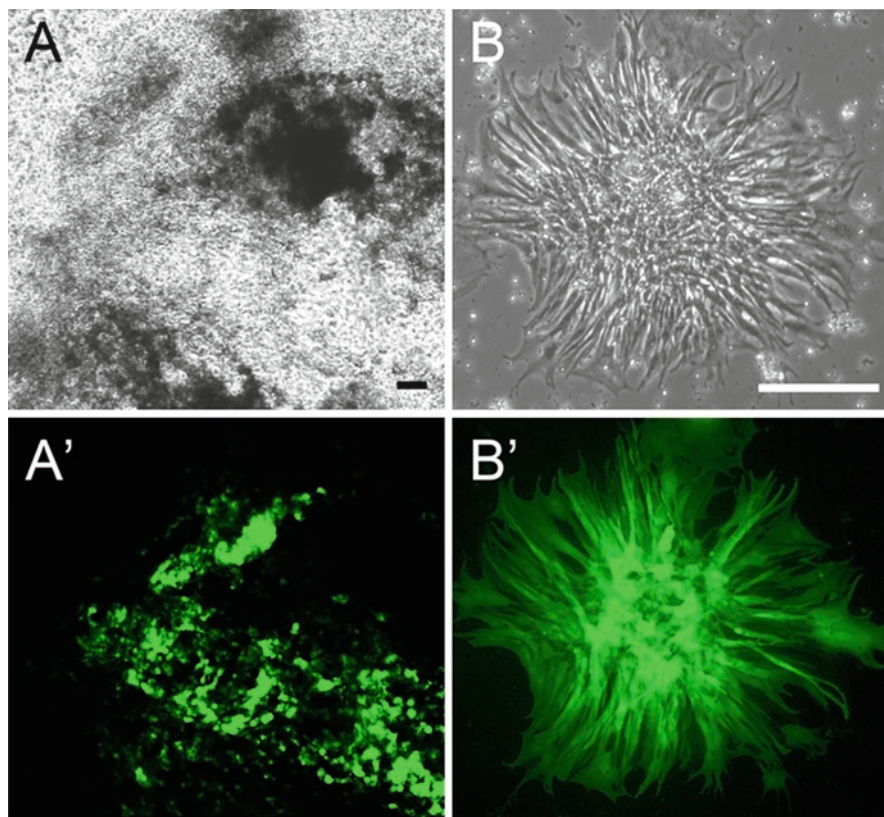
**Table 1** (continued)

Approach	Comments	References
Genetic engineering	Lineage-restricted drug resistance gene resulted in highly purified cardiomyocyte cultures from mouse ESCs	[37, 17, 38]
	Highly purified cardiomyocyte cultures generated by FACS of mouse ESCs expressing a lineage-restricted EGFP reporter	[39]
	Targeted expression of $\alpha$ -1,3-fucosyltransferase enhanced cardiomyocyte differentiation in mouse ESCs	[40]
	Coexpression of EA1, dominant negative p53, and dominant negative CUL7 enhanced cell cycle in mouse ESC-derived cardiomyocytes	[41]
	Expression of SV40 T antigen enhanced cell cycle in mouse ESC-derived cardiomyocytes	[42]
	Antagonization of Wnt/ $\beta$ -catenin enhanced cardiomyocyte differentiation in mouse ESCs	[43]
	Lineage-restricted drug resistance gene resulted in highly purified cardiomyocyte cultures from human ESCs	[44, 45]
Miscellaneous	A single 90-s electrical pulse applied to day 4 EBs increased cardiomyocyte differentiation in mouse ESCs	[46]
	Application of mechanical loading enhanced cardiomyocyte differentiation in mouse ESCs	[47, 48]
	FACS for transient Flk-1 isolated cardiomyogenic progenitors from mouse ESCs	[49]
	Cardiomyocyte enrichment using density centrifugation and cultures of cell aggregates in human ESCs	[50]
	Activin A, BMP4, bFGF, VEGF, and DKK1 treatment, followed by KDR <sup>+</sup> /c-kit <sup>-</sup> FACS, identified cardiovascular progenitor cells in human ESCs	[51]

*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

many studies have reported modest to moderate increases in cardiomyocyte yield in differentiating ESC cultures. Perhaps the most impressive work was from Murry and colleagues [36], who demonstrated that treatment of monolayers of human ESCs with a combination of activin A and bone morphogenetic protein 4, followed by gradient centrifugation, resulted in an average final cardiomyocyte content of 82%. The degree to which this approach can be scaled up for the production of large numbers of donor cardiomyocytes (and, in particular, if directed differentiation is effective in suspension as opposed to in monolayer cultures) remains to be determined.

One of the earliest approaches to enhance cardiomyocyte yield entailed introduction of a lineage-restricted selectable marker. In one example, the cardiomyocyte-restricted MHC promoter was used to target expression of aminoglycoside phosphotransferase (MHC-neo transgene). After spontaneous differentiation, cultures were enriched for cardiomyocytes by simple treatment with G418 [37]. Cultures with more than 99% cardiomyocyte content can routinely be obtained.



**Fig. 3** Adherent culture of EBs generated from ESCs carrying the MHC-EGFP and MHC-neo transgenes after a total of 23 days of differentiation in the absence (**a**, **a'**) or presence (**b**, **b'**) of 11 days of G418 selection. (**a**, **b**) Phase-contrast images; (**a'**, **b'**) epifluorescence images. Note the marked cardiomyocyte enrichment in the G418-treated sample (**b**, **b'**). Bar 100  $\mu$ m

To illustrate this approach, ESCs carrying the MHC-EGFP reporter transgene described earlier as well as the MHC-neo transgene were generated. The ESCs were allowed to differentiate spontaneously, and were then cultured in the absence or presence of G418. In the absence of G418, cardiomyocyte constituted only a small portion of the cultures, in agreement with the data presented above (Fig. 3a). In contrast, G418 treatment effectively eliminated the noncardiomyocytes, resulting in highly enriched cultures (Fig. 3b). This selection approach was readily scalable to bioreactors [52], and could yield more than  $10^9$  cardiomyocytes per 2-L reaction vessel in preparations seeded with dispersed ESC cultures [17]. Similarly, lineage-restricted expression of an EGFP reporter has been employed in conjunction with fluorescence-activated cell sorting (FACS) to generate highly enriched cardiomyocyte cultures [39]. Importantly, both the selection-based and the FACS-based approaches were readily used for the generation of human ESC-derived cardiomyocytes [44, 45, 53].



## 4 Intracardiac Transplantation of ESCs or ESC-derived Cardiomyocytes

Given that the ability to form teratomas in syngeneic or immune-compromised hosts is a major criterion for ESC identification, one would a priori expect that delivery of undifferentiated ESCs into the heart would also give rise to teratomas. Indeed, teratomas were reported following ESC injection into normal [37] or infarcted [54] myocardium. Nonetheless a number of studies have delivered undifferentiated ESCs and failed to report teratoma formation (Table 2). This could reflect compromised differentiation capacity in the ESCs being tested, or alternatively the insufficient

**Table 2** Intracardiac transplantation of ESCs or ESC-derived cardiomyocytes

Donor/host species	Comments	References
Mouse/mouse	Genetically selected cardiomyocytes engrafted in normal myocardium	[37]
Mouse/mouse	In vivo cardiomyocyte differentiation of ESCs required TGF and BMP2	[29]
Mouse/mouse	Intravenous ESC delivery improved cardiac function during viral myocarditis	[55]
Mouse/mouse	Cardiomyocyte-enriched cells plus VEGF enhanced postinfarct function	[56]
Mouse/mouse	Growth factors enhanced ESC engraftment in infarcted hearts	[57, 58]
Mouse/mouse	ESC-seeded synthetic scaffolds improved postinfarct function	[59]
Mouse/mouse	Allogenic ESCs evoked an immune response following heart transplant	[60, 61]
Mouse/mouse	Matrigel enhanced ESC seeding in infarcted hearts	[62]
Mouse/mouse	Genetically selected cardiomyocytes improved postinfarct function	[63]
Mouse/mouse	ESCs improved function in infarcted hearts	[64, 65]
Mouse/mouse	TNF enhanced cardiomyocyte differentiation and lessened teratoma potential	[66]
Mouse/mouse	Cardiomyocytes improved postinfarct function via paracrine mechanisms	[67]
Mouse/mouse	In vivo MR imaging of transplanted cardiomyocytes in infarcted hearts	[68]
Mouse/mouse	Allogenic ESCs formed teratomas when transplanted into infarcts	[54]
Mouse/mouse	Cardiomyocyte engraftment blocked adverse post-MI remodeling	[69]
Mouse/rat	ESC transplantation improved function following myocardial infarction	[70]
Mouse/rat	Density-gradient-enriched cardiomyocytes improve postinfarct function	[71]
Mouse/rat	Differentiated ES cultures survived in immune-suppressed normal heart	[72]
Mouse/rat	ESC-seeded synthetic scaffolds improved postinfarct function	[73]
Mouse/rat	ESCs improved cardiac function in aging hearts	[74]

(continued)

**Table 2** (continued)

Donor/host species	Comments	References
Mouse/rat	GCSF enhanced cardiomyocyte engraftment in infarcted hearts	[75]
Mouse/rat	Intravenously delivered ESCs homed to infarcted myocardium	[76]
Mouse/rat	ESCs formed teratomas when transplanted into infarcted hearts	[77]
Mouse/rat	Chitosan hydrogel enhanced ESC seeding and postinfarct function	[78]
Mouse/sheep	Enriched cardiomyocytes improved postinfarct function	[79, 80]
Human/mouse	Allopurinol/uricase/ibuprofen increased postinfarct cardiomyocyte survival	[81]
Human/mouse	Cardiomyocyte impact on adverse post-MI remodeling is transient	[82, 83]
Human/mouse	KDR progenitors for 3 lineages in vivo improved post-MI function	[51]
Human/rat	In vivo MR imaging of transplanted ESCs	[84]
Human/rat	Microdissected cardiomyocytes improved function in infarcted hearts	[85]
Human/rat	Cardiomyocytes engrafted athymic hearts after ischemia/reperfusion	[86]
Human/rat	Cardiomyocyte engraftment blocked adverse post-MI remodeling	[36, 87]
Human/rat	Cardiomyocytes from BMP2 treatment engrafted infarcted hearts	[88]
Human/rat	ESCs do not form teratomas when engrafted into infarcted hearts	[89]
Human/rat	Physically enriched cardiomyocytes engrafted normal athymic rat heart	[90]
Human/guinea pig	Mixed SAN and cardiomyocyte transplants provided pacemaker activity	[91]
Human/pig	Mixed SAN and cardiomyocyte transplants provided pacemaker activity	[92]

*MR* magnetic resonance, *MI* myocardial infarction, *GCSF* granulocyte colony stimulating factor, *SAN* sinoatrial node

histologic analyses of the engrafted hearts. It has also been suggested that the milieu of the normal or infarcted heart may be sufficient to drive lineage-restricted differentiation of progenitor cells. Nonetheless, the bulk of available data suggest that this is not the case for transplanted ESCs.

Since the initial observation that ESC-derived cardiomyocytes could successfully engraft recipient hearts [37], a large number of experiments have been performed to examine the impact of injecting ESCs or ESC-derived cardiomyocytes into normal or injured hearts (Table 2). Of note, many of these studies indicated that animals receiving ESCs or ESC-derived cardiomyocytes following experimental injury exhibited superior cardiac function as compared with those which did not receive cells. In almost all instances, cardiac function was not improved in the engrafted hearts. Rather, the process of engraftment appeared to attenuate the

deleterious postinjury ventricular remodeling and concomitant decreases in cardiac function. Similar results have been reported with a number of donor cell types. In particular, work by Dzau and colleagues using mesenchymal stem cells strongly suggests that the benefit of cell transplantation in their studies likely reflects the secretion of proangiogenic and antiapoptotic factors from donor cells [93–97]. Such a mechanism would readily explain how engraftment of a relatively small number of ESC-derived cells could impact function in injured hearts. Unfortunately, studies from the Mummery laboratory suggest that this improvement in postinjury remodeling may be transient in nature [82, 83].

Although there are direct data at the cellular level supporting the functional engraftment of fetal cardiomyocytes in recipient hearts [6], the current data available with ESC-derived cardiomyocytes are more circumstantial in nature. Gepstein and colleagues [92] demonstrated that ectopic pacemaker activity originated at the site of engraftment of human ESC-derived cells following atrioventricular node blockade in swine, consistent with the notion that the donor cells were functionally integrated. Similar results were obtained with guinea pig [91]. Despite these promising observations, it would be prudent to directly assess at the cellular level the ability of ESC-derived cardiomyocytes to functionally integrate following engraftment, as formation of a functional syncytium is an absolute requirement for regenerative repair. This is particularly important for studies wherein human ESC-derived cells promoted better function when engrafted into rodent hearts, as it is not at all clear that human cells can sustain rapid rates for extended periods of time. Indeed, rapid pacing is often used to induce heart failure in larger experimental animals [98].

## 5 Future Challenges

The discussions herein suggest that donor cardiomyocytes likely functionally integrate following transplantation into recipient hearts, that methods are available to eliminate the risk of teratoma formation following transplantation of ESC-derived cardiomyocytes, and that approaches to the large scale generation of ESC-derived cells are in hand. Perhaps the greatest challenge facing the use of ESC-derived cardiomyocytes for myocardial regeneration is the limitation in graft size using current approaches. Arguably the best study to date, by Murry and colleagues [36], utilized a combination of materials to enhance survival of donor cells after engraftment. This intervention permitted on average 4% replacement of an infarct which constituted 10% of the left ventricle (which correlates to only 0.4% of the ventricular mass). Thus, we have a long way to go before we will be able to replace transmural myocardial defects.

A number of approaches can be explored to attempt to enhance graft size. For example, many cardiomyocyte prosurvival pathways have been identified [99]. Targeting these pathways in donor ESC-derived cardiomyocytes, either by genetic intervention prior to cardiomyogenic differentiation or via pharmacologic interventions, may facilitate enhancement of donor cell survival, as exemplified by the work

of Murry and colleagues [36]. Similarly, many pathways which impact cardiomyocyte cell cycle activity have been identified [100, 101]. Once again, genetic modification of the ESCs prior to cardiomyogenic differentiation might permit enhanced growth of the cardiomyocyte grafts. Several recent studies suggest that pharmacologic interventions might also be exploited to enhance cardiomyocyte proliferation, particularly if the engrafted cardiomyocytes are not yet terminally differentiated [102–104]. Tissue engineering approaches may also enhance donor cell engraftment. For example, comparably large myocardial replacement was achieved by surgical attachment of collagen-based casts seeded with neonatal rat cardiomyocytes [105, 106].

An alternative strategy to enhance graft size is to transplant ESC-derived committed cardiomyogenic progenitors as opposed to differentiated ESC-derived cardiomyocytes. This approach is based on the notion that progenitor population may exhibit better survival characteristics, and may also be able to undergo postengraftment expansion, thereby resulting in enhanced graft size. Cardiomyogenic progenitors have been identified in differentiating ESC cultures based on transient expression of the vascular endothelial growth factor 2 receptor [49, 51], Nkx2.5 [107, 108], Isl-1 [109, 110], MESP1 [111], or a combination of OCT4, SSEA-1, and MESP1 [112]. Most of the progenitors have been shown to give rise to endothelial and/or smooth muscle cells, in addition to cardiomyocytes. The presence of vascular progenitors might enhance postengraftment donor cell survival and facilitate graft growth. Indeed, enhanced graft size was noted using progenitors isolated by virtue of Isl-1 expression [113]. Transplantation of ESC-derived progenitors into nonhuman primates [112] has also recently been reported.

Clinical use of established ESC lines will likely require some level of immune suppression. The development of immune suppression protocols used for allogenic cadaveric  $\beta$ -cell transplantation [114] would likely be directly transferable to the transplantation of ESC derivatives. The ability to generate autologous ESCs or ESC-like cells would circumvent the need for immune suppression. Several approaches have been developed to accomplish this, including nuclear transfer [115] as well as the generation of ESC-like cells from spermatogonial [116] and mesenchymal [117] stem cells. The ability to generate induced pluripotent stem (iPS) cells developed by Yamanaka and colleagues [118–121] provides a very powerful approach for the generation of autologous donor ESC-derived cells. Importantly, iPS cells exhibit robust and bona fide cardiomyogenic differentiation [122, 123], and many of the interventions and results described above will likely be directly transferrable to iPS derivatives. The main limitation that will likely affect the clinical use of reprogrammed cells is the time requirements for the reprogramming event(s) and implementation of quality control measures to ensure that the individual lines are competent for differentiation and nontumorigenic.

Collectively the studies reviewed herein raise the hope that ESC-derived cells might be useful for the treatment of heart disease, and specifically for the replacement of lost cardiomyocytes. The field has advanced remarkably since the initial report of successful cardiomyocyte engraftment 16 years ago. Given the influx of talented basic and clinical researchers in the field, it is hopeful that the challenges

limiting the clinical use of ESC-derived cells will be overcome, and that ESC-derived cardiomyocyte (or cardiomyocyte precursor) transplantation will become a viable option for individuals with end-stage heart failure.

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