



State of the Art in Cardiomyocyte Transplantation

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Abstract

While other cell-based therapies for ischemic heart disease are generally understood to operate through indirect, so-called “paracrine” mechanisms of action (e.g., by promoting angiogenesis or attenuating adverse ventricular remodeling), cardiomyocyte transplantation aims to repopulate the infarct scar with functionally integrated new myocardium that will directly contribute to contractile function. Early proof of concept for this “direct remuscularization” strategy came from preclinical studies in rodent models in which neonatal and fetal cardiomyocytes were shown to stably engraft within the infarct scar and mediate beneficial

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M. Ieda, W.-H. Zimmermann (eds.), *Cardiac Regeneration*, Cardiac and Vascular
Biology 4, DOI 10.1007/978-3-319-56106-6_9

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effects. More recently, pluripotent stem cells (PSCs) have drawn considerable attention as a more practical, highly scalable source of cardiomyocytes, and these PSC-derived cardiomyocytes have shown considerable promise in both small- and large-animal models of post-infarct heart failure. In this chapter, we review the status of preclinical studies of cardiomyocyte transplantation, describe the significant remaining barriers to clinical translation, and consider potential target patient populations and study design for a first-in-human study of PSC-derived cardiomyocyte transplantation in ischemic heart disease.

9.1 Introduction

The transplantation of human cardiomyocytes has potential to fundamentally change the practice of cardiology by enabling a transition from treatments aimed at merely attenuating symptoms or disease progression to instead the large-scale replacement of damaged or poorly functioning heart muscle. Early work in the field demonstrated the feasibility of cardiomyocyte transplantation and showed that this intervention could result in the “remuscularization” of myocardial scar tissue in a variety of small-animal models of cardiac injury. While a variety of potentially cardiomyogenic cell types have been explored for this application, pluripotent stem cells (PSCs) have been the focus of particularly intense investigation in recent years, as these cells represent a reliable, essentially inexhaustible source of phenotypically unambiguous cardiomyocytes suitable for transplantation. There has been substantial progress toward understanding the factors that regulate the cardiac differentiation of PSCs, and these advances have translated into significant improvements in the reproducibility, yield, purity, and costs of generating PSC-derived cardiomyocytes (PSC-CMs). Much has been learned from PSC-CM transplantation studies in small- and large-animal models of cardiac injury, and investigators in France recently commenced the first clinical trial testing PSC-derived cardiac progenitors in patients with ischemic cardiomyopathy. That said, a number of important challenges remain that must be overcome if cardiomyocyte transplantation is to move from an experimental concept to mainstream practice. For example, currently available PSC-CMs have an immature phenotype, comparable to fetal cardiomyocytes, and this may limit their ability to safely integrate and improve contractile function. Other concerns that must be addressed include the risk of tumor formation and graft-related arrhythmias, the need to prevent graft cell death and immune rejection, and the requirement for large-scale, clinical-grade PSC-CM manufacturing. The most appropriate delivery mechanism has yet to be determined, with some investigators focusing on the direct intramyocardial injection of cardiomyocytes, while others focus on tissue-engineering approaches aimed at generating cellular patches or even whole organs for implantation. To optimize for successful translation and mainstream clinical adoption, it is essential that these and other uncertainties be resolved as much as possible before testing cardiomyocyte transplantation in human subjects.

9.2 How Might Cell Therapy Improve Cardiac Function?

There are multiple potential mechanisms by which intracardiac cell therapy might exert beneficial effects including (1) direct remuscularization (Fig. 9.1), (2) indirect remuscularization via the activation of host cardiomyocyte cell cycle activity or other endogenous reparative pathways, and (3) non-myogenic effects such as enhanced angiogenesis or attenuated adverse remodeling that do not involve remuscularization but might nonetheless enhance contractile function. Depending on the specific clinical scenario, some of these mechanisms may be more appropriate than others. For example, to replace the scar tissue left by myocardial infarction (MI) with functioning muscle, all three of these mechanisms might prove to be a useful component of therapy. Direct remuscularization might prove particularly useful in patients with end-stage post-MI heart failure and already established scar, because adverse remodeling has already plateaued and the window for indirect effects has

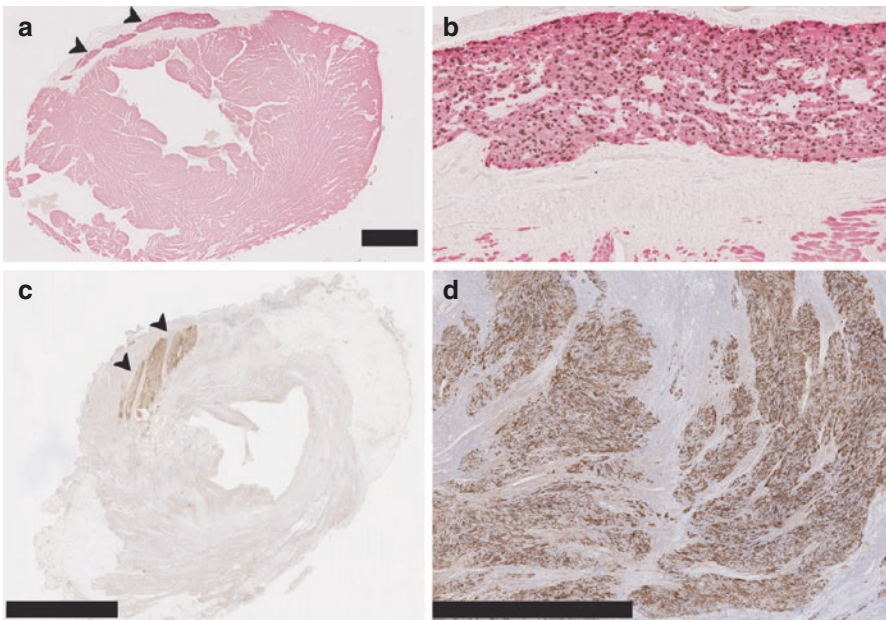


Fig. 9.1 The transplantation of human ESC-derived cardiomyocytes remuscularizes injured guinea pig and nonhuman primate hearts. **(a)** Human myocardium formed within the scar tissue of a cryoinjured guinea pig heart (*arrows*). Host and graft myocardium was identified by immunostaining with an antibody against the cardiac marker beta-myosin heavy chain (*red*), and human nuclei were identified by in situ hybridization with a human-specific pan-centromeric DNA probe (*brown*). Scale bar: 2 mm. **(b)** Inset of graft myocardium. **(c)** Human myocardium formed within the scar tissue of a nonhuman primate heart injured by left anterior descending coronary artery ligation (*arrows*). Graft human myocardium expressing green fluorescent protein (GFP) was identified by immunostaining with an antibody against GFP (*brown*), and host and graft nuclei were identified by hematoxylin stain (*purple*). Scale bar: 5 mm. **(d)** High-power image of graft human myocardium. Staining as in panel C. Scale bar: 4 mm

largely closed. Conversely, a pro-angiogenic cell therapy that increases myocardial perfusion might be expected to be more efficacious in patients suffering from chronic ischemia in the absence of significant scar tissue.

9.2.1 Direct Remuscularization

The delivery of exogenous cardiomyocytes is perhaps the most intuitive of the preceding mechanisms, and early work in the field was nearly exclusively focused on direct remuscularization. By this strategy, cardiomyocytes, cardiomyogenic progenitors, or engineered cardiac tissue are delivered to the injured heart, and they form nascent muscle that must become electromechanically integrated and contract synchronously with host myocardium to augment contractile function. As described in more detail below, early proof of principle for this approach was provided by animal studies in which fetal and neonatal cardiomyocytes were transplanted into a rat model of acute MI (Leor et al. 1996; Li et al. 1996; Scorsin et al. 1997; Scorsin et al. 2000). While these initial reports were limited to showing structural evidence for functional integration (e.g., shared gap junctions between host and graft cardiomyocytes in histological sections), host-graft coupling was later demonstrated directly by imaging genetically tagged grafts loaded with a dynamic fluorescent indicator of graft activation (e.g., calcium-sensitive dyes or fluorescent proteins) (Rubart et al. 2003; Roell et al. 2007). Field and colleagues moved beyond primary cardiomyocytes and demonstrated the possibility of using a more scalable PSC source (Klug et al. 1996). In this work, these authors transplanted cardiomyocytes derived from mouse embryonic stem cells (ESCs) into uninjured mouse hearts and showed that they formed stable intracardiac grafts that persisted for up to 7 weeks (Klug et al. 1996).

9.2.2 Indirect Remuscularization

An alternative approach to direct remuscularization would be to deliver cells that somehow stimulate endogenous cardiac repair, for example, by causing host cardiomyocytes to proliferate and repopulate damaged areas. During mammalian heart development, there is substantial cardiomyocyte proliferation that eventually declines in postnatal life (Pasumarthi and Field 2002; Bergmann et al. 2009). Work in lower vertebrates, which show far greater capacity for cardiomyocyte renewal in the adult heart, illustrates the potential utility of reactivating latent cardiomyocyte cell cycle activity. Indeed, the adult zebrafish heart is capable of large-scale regeneration following apical resection (Poss et al. 2002), and elegant fate-mapping studies have shown that this process involves the proliferation of already-committed cardiomyocytes rather than recruitment of cardiomyogenic progenitors (Jopling et al. 2010; Kikuchi et al. 2010). More recent studies suggest that mammalian hearts are capable of similarly dramatic regeneration in early postnatal life but only during a narrow time window that tracks closely with cardiomyocyte cell cycle activity.

Neonatal mice are able to fully regenerate the cardiac apex following resection at postnatal day 1; however, this ability is lost by postnatal day 7 (Porrello et al. 2011). Interestingly, adult hearts in transgenic mice with cardiomyocyte-restricted overexpression of the cell cycle regulator, cyclin D2, show sustained cardiomyocyte proliferation and robust regenerative capacity following experimental MI (Hassink et al. 2008). While the field continues to explore small-molecule and genetic approaches by which to reactivate cardiomyocyte proliferation in the adult heart, there is some preclinical evidence supporting the notion that the delivery of selected exogenous cell types may also upregulate host cardiomyocyte cycling (Tseliou et al. 2014).

9.2.3 Non-myogenic Mechanisms

Other cardiac cell therapies may be able to exert beneficial effects in the absence of direct remuscularization through indirect, so-called paracrine, mechanisms of action. A number of such effects have been attributed to cell transplantation including increased angiogenesis, decreased cell death, and reduced reverse remodeling (Mirotsou et al. 2011). The largest body of evidence for such indirect beneficial effects following intracardiac cell therapy comes from preclinical and clinical experience with various cell types that can be isolated from the bone marrow (BM) compartment. Indeed, while the field was initially captivated by reports of transdifferentiation and large-scale remuscularization following the transplantation of BM-derived cells (BMDCs), there is now general consensus that these observations can be accounted for by cell fusion and/or experimental artifact (Murry et al. 2004; Balsam et al. 2004; Norol et al. 2007; Iso et al. 2007). By contrast, there is strong evidence that BMDCs can stimulate angiogenesis (Tomita et al. 1999; Nagaya et al. 2004; Tang et al. 2004), reduce reverse remodeling post-MI (Fatkhudinov et al. 2015), reduce apoptosis (Yang et al. 2012), and help host cardiomyocytes re-enter the cell cycle (Matsuura et al. 2004).

9.2.4 Summary

Of course, it still remains to be determined whether any candidate cell type or any of these three broad potential mechanisms of action described above will actually prove efficacious in heart failure patients. Some support for a strategy based on direct remuscularization comes from a recent study that compared the intramyocardial transplantation of human BMDCs, human embryonic stem cell-derived cardiomyocytes (ESC-CMs) and human ESC-derived multipotent cardiovascular progenitors in a rat MI model (Fernandes et al. 2015). In that work, human ESC-CMs and ESC-derived progenitors formed comparable myocardial grafts with similar beneficial effects on left ventricular (LV) systolic function, while human BMDCs promoted angiogenesis and attenuated LV dilatation without engrafting long-term or improving contractility. Since multiple clinical trials suggest that BMDC-based

therapies have only modest if any beneficial effects (Sadat et al. 2014), this preclinical study underscores the need for continued investigation into direct remuscularization as a therapeutic approach for post-MI heart failure.

9.3 What are the Potential Sources of Cardiomyocytes for Transplantation?

9.3.1 Primary Cardiomyocytes

In early proof-of-concept studies, investigators showed that fetal and neonatal cardiomyocytes transplanted into infarcted hearts could survive and mediate beneficial effects on left ventricular contractile function (Leor et al. 1996; Etzion et al. 2001; Scorsin et al. 1997; Li et al. 1996; Skobel et al. 2004; Rubart et al. 2003). In rodent models, fetal cardiomyocytes persist from at least 2 months (Leor et al. 1996; Etzion et al. 2001) to 6 months (Skobel et al. 2004) post-transplantation and couple with host myocardium (Rubart et al. 2003). Their transplantation improves contractile function in multiple models of cardiac injury including cryoinjury (Li et al. 1996), ischemia-reperfusion injury (Scorsin et al. 1997), and ligation of the left anterior descending artery (Etzion et al. 2001; Skobel et al. 2004). Fetal cardiomyocytes appear to improve left ventricular function more than neonatal cardiomyocytes, possibly a consequence of the former's higher proliferation rate post-transplantation (Fujimoto et al. 2011). Successful transplantation and engraftment have also been demonstrated with primary porcine (Watanabe et al. 1998) and human (Leor et al. 1996) fetal cardiomyocytes. While these preclinical studies provided excellent proof of principle for therapeutic remuscularization, it is generally agreed that neonatal and fetal cardiomyocytes are not a practical cell source for clinical application, given their obvious practical and ethical limitations.

9.3.2 Skeletal Muscle

Skeletal and cardiac muscle have many phenotypic properties in common, so skeletal muscle progenitors (myoblasts and satellite cells) drew considerable early attention in the field. This interest was only increased by early reports suggesting that they might be capable of transdifferentiation into functioning cardiomyocytes following intracardiac transplantation (Taylor et al. 1998; Atkins et al. 1999a; 1999b). The latter suggestion was exciting because skeletal and cardiac muscle have very different mechanisms of excitation-contraction coupling and electrical propagation, raising concerns about the capacity of skeletal muscle for electromechanical integration and the possibility of graft-related arrhythmias. Unfortunately, subsequent reports failed to confirm such transdifferentiation phenomena (Murry et al. 1996; Reinecke et al. 2002), and it appears that transplanted myoblasts remain committed to the skeletal muscle phenotype, including lack of

expression of the gap junction protein connexin-43 (Cx43), which is required for function as an electrical syncytium (Scorsin et al. 2000). This outcome may explain in part the findings of the phase II MAGIC trial, in which the patients undergoing autologous skeletal myoblast transplantation showed a signal toward higher mortality and ventricular arrhythmias compared to those receiving placebo (Menasche et al. 2008).

9.3.3 Embryonic Stem Cells

Given their tremendous scalability and undisputed ability to differentiate into phenotypically unambiguous cardiomyocytes, a variety of PSC types have seen intense study in the field in recent years, including embryonic stem cells (ESCs), parthenogenetic stem cells (pSCs), and induced pluripotent stem cells (iPSCs). Human ESCs are isolated from the inner cell mass of blastocysts left over from in vitro fertilization (Thomson et al. 1998). As with all PSCs, ESCs are defined by their capacity for unlimited self-renewal and ability to differentiate into elements from all three embryonic germ layers (endoderm, mesoderm, and ectoderm). When placed into suspension culture in the presence of fetal bovine serum, human ESCs form three-dimensional aggregates called embryoid bodies that include multiple differentiated cell types, including cardiomyocytes (Itskovitz-Eldor et al. 2000; Kehat et al. 2001). Interestingly, different ESC lines show varying potential for “spontaneous” cardiac differentiation under these conditions (Leschik et al. 2015; Moore et al. 2008).

9.3.4 Parthenogenetic Stem Cells (pSCs)

Parthenogenesis is the process of oocyte activation without fertilization by a spermatoocyte (Rogers et al. 2004; Cibelli et al. 2006). This process can be induced by chemical factors in the dish, and the resulting diploid cells can form blastocysts from which pSCs can be isolated. As with their counterparts isolated from normal blastocysts, human pSCs are capable of differentiating into multiple lineages from all the three embryonic germ layers (Lin et al. 2003; Revazova et al. 2007; Brevini et al. 2009; Li et al. 2014; Ahmad et al. 2012; Isaev et al. 2012). While the derivation of cardiomyocytes from human pSCs has not been reported to our knowledge, cardiomyocytes have been successfully obtained from mouse pSCs and employed in preclinical transplantation studies (Didie et al. 2013; Yang et al. 2015). The intriguing potential advantage of pSCs is that they have a largely haploidentical genotype, greatly simplifying their application in major histocompatibility complex (MHC)-matched allotransplantation. That said, a number of concerns have been raised about these cells including their propensity for dysregulated proliferation (Brevini et al. 2009), chromosomal instability (Liu et al. 2011a), and the effects of maternal gene imprinting on differentiation (Stelzer et al. 2011).

9.3.5 Induced Pluripotent Stem Cells

iPSCs are somatic cells that have been reprogrammed into an ESC-like state via the forced expression of transcription factors known to regulate pluripotency. As such, iPSCs offer a number of potential advantages over ESCs: they avoid the ethical concerns associated with the destruction of blastocysts and they could in principle be applied in autologous cell therapies. iPSC-CMs have already reached widespread application for in vitro disease modeling and drug screening (Itzhaki et al. 2011; Sun et al. 2012; Terrenoire et al. 2013; Bellin et al. 2013; Drawnel et al. 2014). That said, there are a number of concerns associated with iPSCs, particularly with regard to clinical applications. iPSCs were initially created by the overexpression of one of two different sets of transcription factors Oct3/Oct4, Sox2, c-Myc, and Klf4 (Takahashi et al. 2007) or Oct4, Sox2, Nanog, and LIN28 (Yu et al. 2007) that were delivered via integrating viral vectors, raising the possibility of random integration events and transformation. These concerns have been greatly allayed by safer reprogramming methods, including the use of non-integrating viral and nonviral vectors, cell-permeant proteins, modified mRNA, and/or small molecules (Zhou and Zeng 2013). Given the phenomenon of epigenetic memory, some investigators have wondered about the long-term phenotypic stability of the differentiation progeny of iPSCs. Finally, at present, individualized patient-specific iPSC-based therapeutics seem impractical from both a regulatory and an economic perspective, greatly undermining the rationale for the use of autologous iPSC derivatives.

9.3.6 Direct Reprogramming to Cardiomyocytes

Another intriguing possibility is the direct reprogramming of fibroblasts (or another readily available somatic cell type) into cardiomyocytes. Just as somatic cells can be reprogrammed into iPSCs via the forced expression of ESC-associated transcription factors, non-myocytes can be induced into a cardiomyocyte-like phenotype via the expression of cardiomyocyte-associated transcription factors. Mouse fibroblasts have been reprogrammed into cardiomyocyte-like cells by expression of Gata4, Mef2c, and Tbx5 (Ieda et al. 2010; Qian et al. 2012), a combination of miRNAs (Jayawardena et al. 2012), and through stimulation with small molecules (Fu et al. 2015). Human fibroblast reprogramming requires a different combination of transcription factors and microRNAs (Nam et al. 2013) but has also been reported. The major barriers to this approach currently are that the reprogramming is very inefficient and that the resultant cells have an incomplete cardiac phenotype (Chen et al. 2012; Qian et al. 2013).

9.4 How Are Human PSC-Derived Cardiomyocytes Obtained and What Is Their Phenotype?

Because PSC-CMs are generally regarded as the most practical, currently available source of cardiomyocytes for transplantation, we focus here on their derivation and phenotype:

9.4.1 Approaches to Cardiomyocyte Derivation from Human PSCs

Tremendous progress has been made toward improving the yield and purity of the cardiomyocytes that can be derived from human PSCs. Initial work in the field involved their differentiation in suspension cultures in the presence of high concentrations of fetal bovine serum. Under these conditions, PSCs can form three-dimensional aggregates, termed embryoid bodies (EBs), that include elements from all three embryonic germ layers (Itskovitz-Eldor et al. 2000). Some EBs contract rhythmically and contain cardiomyocytes, but cardiogenesis by this approach is very inefficient and the resultant cultures are typically comprised of <10% cardiomyocytes (Itskovitz-Eldor et al. 2000; Kehat et al. 2001; Xu et al. 2002). This situation drove the development of more efficient methods to direct the differentiation of PSCs into cardiomyocytes, and numerous protocols have been described that employ signaling molecules known from embryology to be involved in heart development, including Nodal, Wnt, and bone morphogenetic protein 4 (BMP4) (Kinder et al. 1999; Gadue et al. 2005; Naito et al. 2006; Ueno et al. 2007; Yang et al. 2008; Evans et al. 2010; Paige et al. 2010; Zhu et al. 2011; Zhang et al. 2012; Lian et al. 2012; Lian et al. 2013; BurrIDGE et al. 2014). Additional refinements to such methods have been reported involving the manipulation of matricellular cues and/or differentiation under more biomimetic conditions, and there are now multiple protocols that can reach cardiomyocyte purities of >90% with yields of up to 10–40 cardiomyocytes per input undifferentiated cell (Zhang et al. 2012; Lian et al. 2012, 2013; BurrIDGE et al. 2014). Interestingly, some of these methods enable the isolation of multipotent cardiovascular progenitor cells that are capable of giving rise to vascular elements in addition to cardiomyocytes making them potentially useful for cardiac tissue engineering (Yang et al. 2008). Recent efforts to optimize cardiomyocyte differentiation have focused on reducing costs and batch-to-batch variability through the use of small molecules (Lian et al. 2012, 2013; BurrIDGE et al. 2014) while eliminating materials of nonhuman origin (BurrIDGE et al. 2014). These advances have helped pave the way for the large-scale, clinical-grade cardiomyocyte production that would likely be required for therapeutic applications in humans.

9.4.2 Structure and Force Generation

In general, human PSC-CMs are immature and have a phenotype similar to that of cardiomyocytes in the early fetal human heart (Mummery et al. 2003). However, their structural and functional maturation does improve with duration in culture, so our laboratory has found it convenient to separately describe their phenotype at the two stages that have been most frequently examined in the literature (Lundy et al. 2013; Kim et al. 2010; Sartiani et al. 2007; Foldes et al. 2011; Robertson et al. 2013). By this admittedly imperfect schema, so-called “early-stage” cultures refer to human PSC-CMs after approximately 3–5 weeks, while “late-stage” cultures refer to those after >12 weeks of *in vitro* maturation (Lundy et al. 2013). Importantly, human PSC-CMs at both stages are quite distinct structurally from the large (~130 μm long), rod-shaped ventricular cardiomyocytes that populate the adult

human heart (Gerdes et al. 1998). Early-stage human PSC-CMs are small ($\sim 10 \times 15 \mu\text{m}$), round or triangular cells (Mummery et al. 2003; Lundy et al. 2013; Otsuji et al. 2010; Snir et al. 2003), and they have an irregular myofibrillar structure with relatively short sarcomere lengths (Kehat et al. 2001; Mummery et al. 2003; Snir et al. 2003; Lundy et al. 2013). By contrast, late-stage human PSC-CMs are larger, more rectangular in shape (Snir et al. 2003; Lundy et al. 2013; Foldes et al. 2011) and have more and better organized sarcomeres (Lundy et al. 2013), enhanced mitochondrial-sarcomere alignment (Snir et al. 2003), and distinct Z-, A-, H-, and I-bands (Kamakura et al. 2013). Existing human PSC-CMs also have minimal force-generating capacity, with estimates in the range of 1–100 nN per cell (Liu et al. 2012; Taylor et al. 2013; Rodriguez et al. 2014; Yang et al. 2014) versus $\sim 10\text{--}25 \mu\text{N}$ per cell for postnatal cardiomyocytes (van der Velden et al. 1999; Witjas-Paalberends et al. 2013). Somewhat improved measurements have been reported for human PSC-CMs organized into engineered heart tissues using decellularized human cardiac matrix as a scaffold (Guyette et al. 2016), so tissue engineering and/or in vitro electromechanical conditioning may provide a solution to the weak contractile forces of human PSC-CMs.

9.4.3 Electrophysiology

Human PSC-CMs also show gradual maturation in their electrophysiological properties with duration in culture (Mummery et al. 2003; Sartiani et al. 2007; Kim et al. 2010; Lundy et al. 2013), but even late-stage human PSC-CMs are still immature compared to adult cardiomyocytes. Further complicating their electrophysiological characterization, human PSC-CM cultures typically include an admixture of cardiac subtypes (i.e., nodal-, atrial-, and ventricular-like myocytes). As ventricular derivatives are the most appropriate for cardiac repair, we focus on their electrical phenotype here. Compared to adult human ventricular cardiomyocytes, late-stage human ventricular-type PSC-CMs have a less depolarized maximum diastolic potential (MDP), lower action potential (AP) amplitude, slower AP upstroke velocity, and shorter AP duration at 90% repolarization and retain some degree of automaticity (Nabauer et al. 1996; Drouin et al. 1998; Li et al. 1998; Piacentino et al. 2003; Zhang et al. 2009; He et al. 2003; Lundy et al. 2013). The amplitude and kinetics of some important ionic conductances are similar between human PSC-CMs and their adult counterparts, such as the fast sodium (I_{Na}) (Satin et al. 2004; Jonsson et al. 2012; Sheng et al. 2012), the L-type calcium (I_{CaL}) (Zhu et al. 2009; Jonsson et al. 2012), transient outward potassium (I_{to}) (Cordeiro et al. 2013), and delayed rectified potassium (I_{Kr} and I_{Ks}) (Jonsson et al. 2012) currents. However, other important currents show major differences that contribute to their immature AP phenotype. In particular, human ESC-CMs show relatively low levels of the inward rectifier potassium current (I_{K1}) (Sartiani et al. 2007; Cordeiro et al. 2013), which stabilizes the resting membrane potential and controls excitability, and relatively high levels of the hyperpolarization-activated pacemaker current (I_{f}) (Sartiani et al. 2007; Weisbrod et al. 2013). The net result is a propensity for automaticity or

spontaneous electrical activity, and correcting these current densities may result in a more adult-like AP phenotype (Bett et al. 2013; Meijer van Putten et al. 2015). Human PSC-CMs also exhibit slow conduction velocities (only a fraction of that measured in monolayers of postnatal cardiomyocytes (Caspi et al. 2009; Mehta et al. 2011; Lee et al. 2012; Valderrábano 2007)), an outcome that may reflect impaired connexin expression and/or function, as well as isotropically distributed gap junctions.

9.4.4 Excitation-Contraction Coupling

Excitation-contraction coupling in adult ventricular cardiomyocytes involves the opening of voltage-dependent L-type calcium channels and trans-sarcolemmal calcium entry, which is then amplified by calcium-induced calcium release (CICR) from sarcoplasmic reticulum (SR) stores (Bers 2002). As in mature ventricular myocytes, human PSC-CMs exhibit CICR from SR calcium stores that operates by a stringent “local control” mechanism during excitation-contraction coupling (Zhu et al. 2009; Zhang et al. 2013a). However, human PSC-CMs lack transverse tubules (Lieu et al. 2009) and have slow SR calcium reuptake compared to adult cardiomyocytes (Lundy et al. 2013). One explanation for impaired calcium handling in early-stage PSC-CMs may be deficient calsequestrin expression, as overexpression of this calcium-binding protein of the SR has been reported to improve calcium handling in human PSC-CMs (Liu et al. 2009).

9.4.5 Metabolism and Mitochondria

During heart development, cardiomyocytes initially use glycolysis for primary energy production but then later transition to fatty acid metabolism (Fisher 1984; Lopaschuk et al. 1991), and this shift is accompanied by a substantial increase in mitochondrial content (Schaper et al. 1985). Mitochondrial number and volume increase as human PSCs differentiate into cardiomyocytes, but only about one-third of the levels seen in adult ventricular cardiomyocytes (Kerscher et al. 2015; Yang et al. 2014; Schaper et al. 1985). With prolonged duration in culture, human PSC-CMs do show increased fatty acid utilization (Kuppusamy et al. 2015) and show a more adult-like functional localization of mitochondria adjacent to myofibrils (Snir et al. 2003; Lundy et al. 2013).

9.5 How Can the Maturation of PSC-Derived Cardiomyocytes Be Improved?

The maturation of PSC-CMs improves with prolonged duration in culture, but this approach is tedious and expensive and would likely be impractical for large-scale cardiomyocyte manufacturing. Fortunately, there are a number of more scalable

approaches that have shown to promote PSC-CM maturation, at least by some parameters. For example, PSC-CMs treated with the thyroid hormone, triiodothyronine (T3), hypertrophy, elongate, and exhibit longer sarcomere lengths, more mature calcium handling, greater force generation, and increased mitochondrial respiratory capacity (Yang et al. 2014). PSC-CMs also hypertrophy following stimulation with angiotensin II and phenylephrine (Foldes et al. 2011), while treatment with neuregulin promotes electrical remodeling toward a more mature ventricular phenotype (Zhu et al. 2010). Mitochondrial localization improves in PSC-CMs treated with isoproterenol and oleic acid (Chan et al. 2015). Overexpression of let-7-g or let-7i miRNAs accelerates maturation according to structural indices, gene expression profiles, and metabolic parameters (Kuppusamy et al. 2015).

Tissue-engineering approaches have also shown considerable promise for enhancing cardiomyocyte maturation. Human PSC-CMs cultured in three-dimensional environments exhibit improved structural and electrophysiological maturation relative to cells maintained in two-dimensional environments (Zhang et al. 2013b). Cyclical pacing or stretching of these three-dimensional constructs augments the maturation of human PSC-CMs in terms of gene expression, calcium kinetics, conduction velocity, sarcomere organization, and maximal force generation (Nunes et al. 2013; Mihic et al. 2014; Ruan et al. 2015; Hirt et al. 2014). In addition, culture of human PSC-CMs on certain scaffolds improves calcium handling and cell alignment (Khan et al. 2015). Tissue engineering in combination with the maturation methods described above may provide an economical means of maturing human PSC-CMs that could be incorporated into large-scale manufacturing.

9.6 What Have We Learned from Cardiomyocyte Transplantation in Small-Animal Models?

Inspired by previous successes in the field with the transplantation of fetal rodent cardiomyocytes (Leor et al. 1996; Etzion et al. 2001; Skobel et al. 2004; Rubart et al. 2003; Scorsin et al. 1997; Li et al. 1996) and mouse ESC-CMs (Naito et al. 2004; Kolossov et al. 2006; Cai et al. 2007), we and other investigators began human ESC-CM xenotransplantation studies into mice and rats (Kehat et al. 2004; Laflamme et al. 2005; Xue et al. 2005). In early work, we found that human ESC-CMs survived relatively poorly following transplantation into recently infarcted rat hearts but that their engraftment could be significantly enhanced by transiently heat-shocking the cells pre-transplantation and/or delivering them in a pro-survival “cocktail” of factors to improve retention and attenuate cell death (Laflamme et al. 2007a). With these interventions, we were able to remuscularize a significant fraction of the infarct scar in an athymic rat model of subacute MI and observed significant improvements in left ventricular dimensions and global contractile function (Laflamme et al. 2007a). Comparable salutary effects were subsequently reported by other investigators following the transplantation of human ESC-CMs in rodent models of acute or subacute MI (Caspi et al. 2007a, Cai et al. 2007; van Laake et al.

2007), although not all found these benefits to be durable to later time points (van Laake et al. 2007; 2008). Interestingly, in a rat model of chronic MI with already established scar tissue and ventricular dysfunction, we found that human ESC-CMs engraft but do not mediate significant beneficial effects on contractile function (Fernandes et al. 2010).

Less has been reported regarding the transplantation of human iPSC-CMs in animal models, but the best available data suggest that human iPSC-CMs and ESC-CMs have comparable phenotypes *in vitro* (Zhang et al. 2009; Lundy et al. 2013), so one might also expect comparable outcomes *in vivo*. A recent study in a rat MI model compared outcomes following the transplantation of human iPSC-CMs after 8, 20, and 30 days of *in vitro* differentiation (Funakoshi et al. 2016). These authors found that the optimal graft size was obtained with the transplantation of 20-day-old cultures, suggesting this may be an optimal time point for harvesting and implantation. Encouragingly, they found durable beneficial effects on left ventricular dilation and fractional shortening up to 12 weeks post-transplantation.

For true heart regeneration, the newly formed muscle must be capable of electromechanical integration and synchronous activation with host myocardium. Human ESC-CMs have the subcellular machinery necessary for such integration (e.g., gap junctions), and early mapping studies provided indirect evidence that they could couple with host tissue following transplantation in uninjured hearts (Kehat et al. 2004; Xue et al. 2005). To directly demonstrate whether human ESC-CMs were capable of 1:1 host-graft coupling following transplantation into injured hearts, our group turned to the guinea pig (Fig. 9.1 panels a, b), because this small-animal model has a sinus rate (Shiotani et al. 2007) that is much closer to that of humans than do mice and rats (Swoap et al. 2004). For these experiments, we created transgenic human ESC-CMs that stably expressed GCaMP3, a calcium-sensitive fluorescent protein. *In vitro* these myocytes show robust fluorescence transients with each cycle of contraction. Hence, by imaging the epicardial surface of hearts engrafted with GCaMP3⁺ human ESC-CMs, we can use the graft-autonomous GCaMP3 fluorescent signal to determine whether the graft tissue is electrically active and, if so, the temporal relationship of its activation to that in host myocardium. By this approach, we found that 100% of human GCaMP3⁺ ESC-CM grafts in uninjured guinea pig hearts were 1:1 coupled with host myocardium (as indicated by a 1:1 relationship between GCaMP3 fluorescent transients and QRS complexes of the host ECG). Applying the same approach to human GCaMP3⁺ ESC-CMs transplanted in a guinea model of subacute MI, we observed reliable 1:1 host-graft coupling in a majority (~60%) of recipient hearts, proving that the newly formed graft muscle is at least capable of electromechanical integration with host myocardium (Shiba et al. 2012). More recently, we found that foci of 1:1 host-graft coupling can also be found in a guinea pig model of chronic MI but that the extent of electromechanical integration is significantly reduced in this context (Shiba et al. 2014).

In summary, much important information was gleaned from the preceding work in small-animal models of MI. First, PSC-CMs, including human ESC-CMs, can engraft in injured hearts and form stable implants of electromechanically integrated new myocardium within the infarct scar, a *sin qua non* for true heart regeneration.

Second, teratoma formation has not been reported, at least when the implanted PSC-CMs were of sufficient cardiac purity. Finally, most studies have reported beneficial effects on left ventricular contractile function, at least attenuating the adverse remodeling and progressive loss of fractional shortening that otherwise occurs over time. Taken collectively, this body of work laid the groundwork for the large-animal studies discussed in the following section.

9.7 What Have We Learned from Large-Animal Studies?

Although not necessarily a prerequisite for regulatory approval prior to human clinical trials, large-animal models provide important information that cannot be obtained using small-animal or *in vitro* models. Large animals possess a heart size and rate similar to humans (Gandolfi et al. 2011). Differences also continue down to the cellular and molecular levels (for a detailed review, see Chong and Murry 2014). Large-animal models also enable the testing of clinical routes of cell delivery that are not feasible in small-animal models, such as intracoronary and trans-endocardial injection (discussed further below).

Kehat and colleagues were the first to use human ESC-CMs in a large-animal model study to test their capacity for electromechanical integration following transplantation into normal (i.e., uninjured) intact myocardium (Kehat et al. 2004). In a porcine model of heart block, human ESC-CMs engrafted, integrated with the host, and provided a new source of ventricular electrical activation (as evidenced by electrocardiographic recordings and electroanatomic mapping studies). This pioneering work heralded the possibility of “biological pacemakers” composed of human PSC-CMs that could 1 day replace the implantation of mechanical devices. Importantly, this large-animal model enabled the use of a highly specialized human clinical electroanatomical mapping system to localize the source of the new ventricular ectopic rhythm. Catheter-based mapping studies of this nature would be impractical in small-animal models.

Nonhuman primates have high genetic conservation with humans and therefore provide important information for what could be expected with human cardiomyocyte transplantation. Blin and colleagues investigated the intracardiac transplantation of allogeneic nonhuman primate ESC-derived cardiovascular progenitors defined by the expression of the cell surface marker stage-specific embryonic antigen-1 (SSEA-1 or CD15) and showed that these cells help repair and regenerate infarcted recipient hearts (Blin et al. 2010). After treatment with BMP2 and WNT3A to form cardiac mesoderm, 20 million SSEA-1-positive cardiovascular progenitors were purified by magnetic bead sorting and injected using direct visualization into rhesus monkey heart infarcts. Interestingly, only calcineurin inhibitors (FK506 or cyclosporine) were used for immunosuppression in this allogeneic approach. Small grafts that express cardiomyocyte, smooth muscle, and endothelial cell proteins were present at 2 months; however, no cardiac functional assessment was performed.

In a different approach, SSEA-1-positive nonhuman primate ESC-derived cells were combined with autologous adipose-derived mesenchymal stromal cells and

arranged into sheets (Bel et al. 2010). These combined cell sheets were surgically implanted onto the epicardial surface of infarcted monkey hearts and showed donor-derived cardiomyocyte differentiation at 2 months. Although it was not technically possible to distinguish, the few cardiomyocytes most likely came from the ESC-derived cells rather than the mesenchymal cells within the delivered sheets. Following cell sheet delivery, there was a trend toward improved left ventricular function; however, the small number of animals examined precludes statistically robust conclusions.

Our group recently used a nonhuman primate model to demonstrate that human ESC-CMs can electromechanically couple and remuscularize the infarcted heart (Fig. 9.1 panels c, d) (Chong et al. 2014). In contrast to previous studies, we delivered a much higher cell number (1 billion cardiomyocytes per heart) via trans-epicardial injection under direct visualization following a mini-thoractomy. The immunosuppression regimen consisted of prednisolone, cyclosporine, and the T cell co-stimulatory blocking antibody, abatacept. Using this approach, we observed considerably larger cardiomyocyte grafts than other studies. To demonstrate electromechanical coupling, we used the same method as in the guinea pig model above to prove integration. Importantly, all infarcted monkeys receiving human ESC-CMs showed ventricular tachyarrhythmias by telemetric electrocardiographic monitoring, events that were not observed prior to cell transplantation or in infarcted animals receiving vehicle alone. The mechanistic basis for these arrhythmias remains undefined and will be crucial to determine prior to progression to clinical studies.

Recently, a porcine myocardial infarction model has been used to study reparative effects of human iPSC-derived cells (Ye et al. 2014). A unique tissue-engineering approach was employed involving fibrin patches containing insulin-like growth factor-1 (IGF-1) encapsulated microspheres. These patches were implanted alone or followed by trans-epicardial injection of a combination of 2 million human iPSC-CMs, endothelial cells, and smooth muscle cells (6 million cells in total). The fibrin patches enhanced cell engraftment and cardiac functional improvement. Notably, no ventricular arrhythmias were observed in this study. For the telemetry portion of the study, animals received the patch alone or followed by human iPSC-CM transplantation. It remains unclear if the lack of arrhythmias is due to a smaller graft size, treatment with IGF-1, or if arrhythmias would have been observed after cardiomyocyte transplantation without a fibrin patch.

The preceding large-animal studies provided excellent proof of concept for cardiomyocyte transplantation as well as information of high clinical relevance. Notably, the large-scale production of useful cardiovascular derivatives from PSCs is feasible, although additional refinements will be required to improve the efficiency and costs of cell manufacturing, as well as compliance with the good manufacturing practice guidelines required for clinical use. The lack of tumorigenicity seen in small-animal studies was replicated in larger animals despite the delivery of much larger cell doses in some studies. More ominously, our group observed graft-related arrhythmias following human ESC-CM transplantation in infarcted primate hearts. This phenomenon was not seen in early small-animal studies with the same cells, underscoring the need for additional work in large-animal models with slower

heart rates and greater cardiac mass. These and other important issues that will need to be addressed before clinical trials can be efficiently run are considered in the following sections.

9.8 What Are the Remaining Challenges Associated with Cardiomyocyte Transplantation?

9.8.1 Cost, Scalability, and Production

The adult human heart contains ~4 billion cardiomyocytes (Murry et al. 2006), and the loss of one-quarter of these myocytes is known to result in heart failure (Caulfield et al. 1976). Hence, while detailed dose-response experiments will be required to empirically determine the precise number of cells required, we anticipate that a cell therapy predicated on the repopulation of infarcted myocardium would require the stable engraftment of ~1 billion cardiomyocytes. In the specific case of PSC-CM-based therapies, this cell number target would obviously need to be adjusted to account for losses due to graft cell death (Robey et al. 2008) and/or immune rejection (as discussed below). While these numbers are approximately two orders of magnitude larger than the number of cells that have been employed during earlier clinical experience with BMDCs, our group and others have shown the feasibility of human PSC-CM manufacturing at this scale, even with existing cardiac differentiation methods (Chong et al. 2014). One mitigating factor is that human PSC-CMs can be cryopreserved, banked, and then thawed at high viability just prior to transplantation (Xu et al. 2011).

It is generally accepted that suspension culture systems will likely be required for cardiomyocyte manufacturing on the scale needed for human patients. The large-scale growth of undifferentiated human PSCs has been demonstrated with up to 1 billion PSCs per flask (Chen and Couture 2015). Laminin microcarriers can potentially further improve the yield of undifferentiated human PSCs in culture and have the added benefit of significantly reducing media cost (Lam et al. 2015). While variability remains in cardiomyocyte yield, some microcarrier-based systems have reached 0.3–0.6 cardiomyocytes per input PSC (Lecina et al. 2010). Of course, cardiomyocyte purity is also a concern, and with cyclic perfusion feeding, human PSC-CMs can be produced at 85% purity (Kempf et al. 2014).

Any PSC line used in human patients will have to be cultured and differentiated using clinical-grade, good manufacturing practices (GMP). To date, there are only a handful of human ESC lines that have been produced under GMP conditions (Prowse et al. 2014). Only three human ESC lines (HAD-C 100, 102, and 106) have been reported that were derived using both GMP and xeno-free conditions (Tannenbaum et al. 2012). While it is not clear that animal-free conditions will be absolutely required for regulatory approval in humans, it is clearly desirable, given persistent concerns about the infectious risks of interspecies transplantation. Importantly, whichever human PSC line is selected for clinical-grade production of cardiomyocytes for human transplantation, robust and reproducible cardiomyocyte

differentiation will have to be confirmed in that cell line along with repeating some basic preclinical studies to demonstrate the expected behavior following transplantation.

9.8.2 Graft Survival

It has been estimated that >90% of graft cardiomyocytes die shortly following intracardiac transplantation (Reinecke et al. 1999), an outcome that limits the extent of remuscularization obtained unless the initial cell dose can be increased proportionately. While some cell loss can be recovered through proliferation post-transplantation, human PSC-CMs show only modest cell cycle activity that tapers off over time (Laflamme et al. 2005, 2007a, Caspi et al. 2007a). This situation has led to intense interest in identifying the mechanisms of cell death and novel methods to overcome them. In addition to delivery into the poorly vascular and pro-inflammatory environment of the infarct scar, it is generally accepted that multiple pathways contribute to graft cell death and that a multifactorial approach will likely be required (Robey et al. 2008). For example, in our own early studies of human ESC-CM transplantation in infarcted rat hearts, we found that, while individual interventions were insufficient, engraftment could be improved by combining heat-shock, provision of matricellular attachment factors, and pharmacological inhibition of multiple mitochondrial and caspase-mediated cell death pathways (Laflamme et al. 2007a). A number of other effective pro-survival interventions have been reported with ESC-CMs, including delivery in aggregates rather than as dissociated single-cell suspensions (Moon et al. 2013), activation of cytoprotective heme oxygenase-1 activity by cobalt protoporphyrin pretreatment (Luo et al. 2014a; Luo et al. 2014b), and co-delivery with supportive stromal cells (Xi et al. 2011). Transplantation studies with primary cardiomyocytes and/or other myogenic cell types have suggested other possibilities including delivery in collagen hydrogels (Kutschka et al. 2006a), Bcl-2 overexpression (Kutschka et al. 2006b), overexpression of specific miRNAs (Hu et al. 2011; Liu et al. 2011b), and removal of the pro-fibrotic and antiangiogenic matrix protein, thrombospondin-2 (Reinecke et al. 2013).

9.8.3 Immune Rejection

It is well known that transplantation of cells or tissue, foreign to the host, results in an immune reaction and this has been observed with ESC-CM transplantation (Dai et al. 2007). Therefore, either preventing immune rejection via pharmacological immunosuppression and recipient-donor human leukocyte antigen (HLA) matching or evading the immune system altogether will be key components of a viable cardiomyocyte transplantation therapy. While the risks inherent in immunosuppression (including increased propensity for infection and malignancy) are deemed acceptable in end-stage heart failure patients being listed for whole-organ transplantation,

this calculation would not necessarily apply in less severely ill individuals. Indeed, the need and nature of the immunosuppression associated with any given cell therapy will inevitably affect the type of patient for whom such therapy would be appropriate. Importantly, immunosuppression in heart transplant recipients is tailored over time based on pathological evaluation of surveillance biopsies obtained via catheter from the right ventricle. Because the graft myocardium formed following cell transplantation is unlikely to be similarly accessible to surveillance biopsy, it will be challenging to similarly “fine-tune” immunosuppressant therapy in cell therapy recipients.

At first glance, an attractive approach would be to use autologous cells for transplantation, for example, using patient-derived iPSCs in each and every patient. Unfortunately, in our opinion, this strategy is completely impractical at present. While the methods of reprogramming have become more standardized and efficient over time, the costs remain prohibitively high, and it takes weeks to months to generate iPSCs and their cardiac derivatives. Equally importantly, it is not clear that there is a regulatory structure in place to approve such individualized cell therapies, even if the economic and quality-control issues could be satisfactorily resolved (Condic and Rao 2008; Solomon et al. 2015).

An alternative approach is to create and maintain large banks of genetically diverse PSCs that would allow for HLA matching (Taylor et al. 2005; Nakajima et al. 2007), much as is done currently in solid-organ transplantation. Recognition of HLA class I molecules by T lymphocytes may be the primary mechanism for immune rejection of transplanted human ESCs (Deuse et al. 2011). HLA class I molecules are expressed on most nucleated cells (Karabekian et al. 2015), and HLA I matching could help reduce immunosuppression requirements following transplantation. To reduce the number of cell lines needed in such a repository for whole population matching, one could turn to HLA-homozygous human pESCs (Daughtry and Mitalipov 2014). That said, it is probably unnecessary to consider ABO blood group antigens for patient matching, since these antigens are lost after human ESCs differentiate into cardiomyocytes (Molne et al. 2008).

A more exotic but attractive alternative approach would be to create a universal donor line that has been engineered to evade the immune system. Targeting HLA class I molecule-mediated T lymphocyte activation holds some promise. Cytotoxic T lymphocyte antigen 4 (CTLA4) binds to CD80 and CD86 preventing T cell co-stimulation by CD28, whereas programmed death ligand-1 (PD-L1) directly inhibits T cell activity (Rong et al. 2014). Human ESC-CMs that express CTLA4-Ig and PD-L1 survive when transplanted into the hind limbs of non-immunosuppressed mice with humanized immune systems (Rong et al. 2014). Preventing T cell activation improves human ESC-CM graft survival in ischemic mouse myocardium (Huber et al. 2013). Beta-2 microglobulin is a component of the invariable, non-transmembrane light chain of HLA class I molecules (Karabekian et al. 2015). Human ESCs with disrupted beta-2 microglobulin have impaired surface expression of HLA class I molecules and reduced immunogenicity in vitro (Lu et al. 2013a), and beta-2 microglobulin-reduced human ESC-CMs do not stimulate allogeneic T cell proliferation in vitro

(Karabekian et al. 2015). If these strategies are successful in creating a universal donor human ESC, it would have tremendous impact on advancing the field of cardiomyocyte transplantation.

9.8.4 Integration and Arrhythmias

To contribute useful new force-generating units, graft myocardium must integrate and contract synchronously with host muscle. As described above, human ESC-CM grafts have been shown to be capable of such electromechanical integration in both intact and recently injured hearts (Kehat et al. 2004; Shiba et al. 2012; Shiba et al. 2014; Chong et al. 2014; Ye et al. 2014). Their capacity for integration in chronically injured hearts with established scar has been less well studied, but the best available evidence suggests that their integration is much more limited in this context, perhaps accounting for their failure to exert beneficial effects on contractile function in a guinea pig chronic MI model (Fernandes et al. 2010). If this observation is born out in subsequent large-animal studies, it would represent a significant barrier to successful translation in the large proportion of patients with remote MIs and established heart failure.

The capacity of PSC-CMs to integrate is a double-edged sword. While electrical coupling between graft and host tissue is required to mediate contractile benefits in a direct remuscularization strategy, it raises concerns about the risk of graft-related arrhythmias. Indeed, in the aforementioned work by our group in a nonhuman primate MI model, we observed nonlethal ventricular tachyarrhythmias in 100% of infarcted primates receiving hESC-CMs (Chong et al. 2014). There are several potential mechanisms by which PSC-CMs could contribute to electrical instability. One possibility is graft ectopy, i.e., a situation in which the graft tissue acts as an ectopic pacemaker. Consistent with this mechanism, most available PSC-CMs are comprised of a mixture of cardiac subtypes including a significant fraction of nodal/pacemaker myocytes (Zhang et al. 2012; Karakikes et al. 2014; He et al. 2003; Zhu et al. 2010; Moore et al. 2008; Kamakura et al. 2013; Weng et al. 2014). Moreover, ventricular myocytes in the developing heart show some degree of automaticity, so even purified ventricular PSC-CMs would be expected to retain some degree of automaticity unless matured (Zhang et al. 2002; Sartiani et al. 2007; Kim et al. 2010). Tachyarrhythmias may also arise from slowly firing ectopic foci within the infarcted area because of poor coupling, source-sink electrical mismatch, or network heterogeneity (Pumir et al. 2005; Biktashev et al. 2008). PSC-CMs have also been reported to have a propensity for afterdepolarizations and triggered arrhythmias (Zhang et al. 2002). Finally, PSC-CM graft tissue also shows low levels of isotropically distributed connexin-43, a situation that likely underlies the slow conduction velocities recently measured in graft tissue (Shiba et al. 2012, 2014; Chong et al. 2014). Slow conduction velocity, particularly through spatially heterogeneous islands of poorly connected graft tissue, seems likely to promote reentrant arrhythmias (Shiba et al. 2012, 2014). Overall, this is an understudied area, and the atypical ion channel expression and diverse mechanisms of graft-related arrhythmia may make them refractory to conventional antiarrhythmic therapies.

9.8.5 Tumorigenicity

PSCs are defined by their ability to form teratomas (a benign proliferation including differentiated elements from all three embryonic germ layers), so the risk of tumor formation is an inherent concern with any PSC-based cell therapy. The risk of tumor formation with any given therapy will obviously vary with cell dose, graft-cell autonomous factors (e.g., proliferative index), host factors (e.g., graft environment, host immune responses, inflammation), and the route of delivery. Cell dose is a particular issue for cardiovascular applications in which relatively large cell numbers ($\sim 10^9$ per recipient) are likely to be required.

There are two sources from which tumors may arise following PSC-based therapies: (1) residual undifferentiated PSCs that might give rise to teratomas (Nussbaum et al. 2007; Fico et al. 2012; McLenachan et al. 2012; Menendez et al. 2012) and (2) malignant transformation of graft cells, which might lead to tumors even in the absence of undifferentiated cells (Ben-David et al. 2014). To minimize the risk of the first possibility, it is obviously desirable to maximize cardiomyocyte purity while minimizing or eliminating undifferentiated PSCs or incompletely committed PSC derivatives. Undifferentiated cells can be depleted by cytotoxic targeting of the cell surface marker claudin-6 (Ben-David et al. 2013), inhibition of stearyl-coA desaturase (Zhang et al. 2014), or culture under methionine-free conditions (Matsuura et al. 2015). A number of strategies to increase PSC-CM purity have been described including Percoll gradient centrifugation (Lin et al. 2010), promoting differentiation of cardiac progenitor cells by TNF α overexpression (Behfar et al. 2007), sorting via expression of SIRPA (Dubois et al. 2011) or VCAM-1 (Ponten et al. 2013), mitochondrial labeling (Hattori et al. 2010), and metabolic selection with glucose depletion (Hemmi et al. 2014). Simply increasing the time in culture post-differentiation may also reduce or eliminate tumorigenicity as has been shown to be the case with neural precursors (Kozubenko et al. 2010; Doi et al. 2012a, b; Kobayashi et al. 2012). Collectively, the enrichment of human PSC-CM purity and time in culture post-derivation may account for their tumor-free engraftment observed in mouse (van Laake et al. 2008), rat (Caspi et al. 2007a; Citro et al. 2014), guinea pig (Shiba et al. 2012, 2014), pig (Ye et al. 2014), and nonhuman primate (Chong et al. 2014) models. With regard to the risk of malignant transformation of graft cells, the main factor is likely aneuploidy that can occur with prolonged cell culture (Ben-David et al. 2014). This risk can be mitigated through careful monitoring of human PSCs and their derivatives for karyotypic abnormalities during cell manufacturing. Cardiomyocytes themselves have a relatively low cell cycle activity (perhaps accounting for the relatively rarity of primary cardiac tumors), so the risk of transformation is probably relatively low once PSCs have become cardiac-committed.

9.9 How Should Cardiomyocytes Be Delivered to the Myocardium?

Depending on the clinical requirements, there are different therapeutic approaches to cardiomyocyte transplantation that could be considered. For example, if cell therapy must be administered emergently during an acute MI, then the transplant

material has to be readily available, i.e., available “off the shelf”. Many preclinical experiments performed thus far have used injection of whole-cell suspensions to repair damage from a MI, an approach that can be extrapolated to an allogeneic, “off-the-shelf” cell product. On the other hand, this approach, involving the local delivery of therapeutic cells, may be inappropriate in other clinical contexts, for example, a non-ischemic cardiomyopathy in which all or nearly all of the myocardium is dysfunctional. In this situation, tissue engineering or whole-organ fabrication may be needed to wholly replace the diseased tissue. These latter methods would also be amenable to incorporation of biomaterials aimed at structural support or providing cellular maturation cues.

The delivery mechanism that has been employed in the vast majority of preclinical studies of human PSC-CMs to date is direct, transepical cell injection via an open thoracotomy (Shiba et al. 2014; Chong et al. 2014). Because this approach requires exposure and direct visualization of the targeted region of the heart, it is necessarily invasive. Hence, unless less-invasive strategies (i.e., a mini-thoracotomy) can be demonstrated, this transepical route will require a sternotomy in humans, making individuals that already have an indication for cardiac surgery an attractive patient population for early clinical trials. A number of other less-invasive and even closed-chest approaches to cardiomyocyte delivery have been proposed, including steerable catheters with an injection needle for endocardial delivery or intracoronary catheters equipped with an injection needle (Wang et al. 2009). Steerable endocardial catheters could be guided using fluoroscopy (Amado et al. 2005), echocardiography, or voltage mapping as is done with cardiac electrophysiology procedures. Another benefit of direct myocardial injection over intracoronary delivery is that materials, such as hydrogels, can be delivered in addition to cardiomyocytes to provide a biodegradable scaffold during engraftment (Habib et al. 2011).

While a number of adult stem cell types have been delivered to the myocardium via an intracoronary route including autologous cardiosphere-derived cells (Makkar et al. 2012; Malliaras et al. 2014) and non-myogenic stem cells (Katritsis et al. 2005; Yang et al. 2010), this approach is not likely relevant to the delivery of PSC-CMs. First, the retention of cells following delivery via the intracoronary route is known to be relatively poor (with ~2% of the injected cells being retained) (Leiker et al. 2008), obviously problematic in a situation in which billions of stably engrafted cardiomyocytes are required. A more important consideration relates to cell size. Human PSC-CMs ($\geq 25\text{--}40\ \mu\text{m}$ long) (Lundy et al. 2013) are significantly larger than other cell types that have been delivered via the intracoronary route including BMDCs ($\sim 10\ \mu\text{m}$), mesenchymal stem cells ($\sim 20\ \mu\text{m}$) and cardiosphere-derived cells ($\sim 20\ \mu\text{m}$) (Johnston et al. 2009). It is very unlikely that differentiated cardiomyocytes would be capable of diapedesis and migration out of a vessel wall, so their larger size and predilection for clumping poses a major risk for vascular occlusion.

Another approach that has proven promising in animal studies is the delivery of human PSC-CMs after organization into engineered cardiac cell sheets. Early proof of principle for this approach came from studies using primary cardiomyocytes. Cardiac cell sheets are formed by culturing cardiomyocytes in monolayers on temperature-responsive polymers and then releasing the monolayer from the polymer by reducing the temperature (Shimizu et al. 2002). Monolayers can be stacked

together to form multilayer cardiac cell sheets that can be transplanted onto the heart, integrate with host tissue, are angiogenic, and improve cardiac contractile function (Miyagawa et al. 2005; Sekiya et al. 2006; Furuta et al. 2006; Sekine et al. 2006). Importantly, engineered cardiac cells sheets can be made using human iPSC-CMs, and these have been engrafted into a porcine model of myocardial infarction with improved cardiac performance, even though the cells did not survive long term (Kawamura et al. 2012). The thickness of engineered cardiac cell sheets has typically been limited to three layers because of hypoxia; however, sequential grafting or alternating with layers of gelatin hydrogel microspheres can produce sheets between 9 and 15 layers thick (Komae et al. 2015; Matsuo et al. 2015). As an alternative to cardiac cell sheets, human ESC-CMs can be grown on polymer scaffolds or can be mixed with collagen and cast into three-dimensional constructs, termed engineered heart tissue (EHT) (Chen et al. 2010; Xu et al. 2013; Chen et al. 2015; Riegler et al. 2015). EHTs from human ESC-CMs and collagen have been successfully engrafted onto rat hearts in a chronic MI model, and while cardiac systolic function improved, the improvement did not correlate with cardiomyocyte viability (Riegler et al. 2015). Because cardiac cell sheets and EHTs are engineered, different cells and materials can be incorporated to change their function. Changing cellular composition by adding fibroblasts and/or vascular cells can improve vascularization, conduction velocity, or tension of the engineered tissue (Caspi et al. 2007b; Sekine et al. 2008; Lesman et al. 2010; Tulloch et al. 2011; Kreutziger et al. 2011; Liao et al. 2011; Hibino et al. 2012; Thavandiran et al. 2013). Some biodegradable scaffold materials have conductive properties (Baheiraei et al. 2015), are autologous like the omentum (Shevach et al. 2014) and urinary bladder (Turner et al. 2012), or structurally and mechanically tunable like silk combined with cardiac extracellular matrix (Stoppel et al. 2015). Engineered tissue may also present new challenges such as engineered cell sheets being capable of producing reentrant arrhythmias in vitro (Kadota et al. 2013); however, it is not known how host-graft integration will impact this.

Whole-organ tissue engineering is yet another approach to replacing damaged cardiac tissue, but this will be significantly more complicated than constructing small, three-dimensional EHTs. Building the scaffolds from the ground up for this type of fabrication has not yet been accomplished, but cell-free extracellular matrix scaffolds with preserved structure have been made from multiple organs, including the heart, using organ decellularization (Ott et al. 2008; Wainwright et al. 2010; Carvalho et al. 2012; Guyette et al. 2014; 2016; Weymann et al. 2015; Kawasaki et al. 2015). Human iPSC-CMs can grow on cell-free scaffolds made from both human (Oberwallner et al. 2014; Guyette et al. 2016) and mouse myocardium (Lu et al. 2013b). Decellularized porcine heart scaffolds have been reseeded with mixtures of cells including neonatal mouse cardiomyocytes and human umbilical vein endothelial cells (Weymann et al. 2014) as well as neonatal rat cardiomyocytes, fibroblasts, and endothelial cells in rat heart scaffolds (Yasui et al. 2014). For whole-organ tissue engineering to be a successful method of cardiomyocyte delivery, there are important hurdles that remain. One of the most significant problems is that acellular vascular grafts are thrombogenic; and while this phenomenon can be reduced

somewhat by re-endothelialization (Robertson et al. 2014), graft intravascular thrombosis would clearly impair perfusion and graft viability. Seeding efficiency also remains very low, for example, despite the introduction of as many as 500 million human iPSC-CMs into whole human heart cardiac matrices, only 50% of a small ($\sim 5 \text{ cm}^3$) target region was successfully reseeded in one recent study (Guyette et al. 2016). In our opinion, these parameters would have to be greatly improved for this approach to compete successfully with the injection of cardiomyocyte cell suspensions, which have been shown to successfully form comparably sized volumes of host-perfused myocardium (Chong et al. 2014).

9.10 What Preclinical Information Is Still Needed to Facilitate Successful Translation?

The field has made tremendous progress in recent years, but, in the present authors' opinion, there are still many unanswered questions that should be addressed in preclinical studies to optimize cardiomyocyte transplantation for success in eventual clinical trials. Most importantly, we need to take every precaution to avoid an outcome akin to the one that derailed the gene therapy field for many years, and there are a number of major safety concerns (e.g., graft-related arrhythmias, tumorigenesis) that are inherent to cardiomyocyte transplantation. Moreover, we must be mindful that there are finite resources available to support the development and testing of novel cardiovascular therapeutics. Even putting aside the risk of patient harm, a premature move to the clinic and finding a null or insignificant beneficial effect could permanently derail progress in the field. Indeed, there may be recent precedent for this outcome from clinical trials testing BMDCs in ischemic heart disease. BMDCs moved relatively rapidly from small-animal studies to humans, and it has been suggested that their disappointing performance in randomized clinical trials may have resulted in part from an incomplete mechanistic understanding, suboptimal cell doses and timing of delivery, and an inappropriate target patient population (Laflamme et al. 2007b; Simari et al. 2014). Given that PSC-based therapies arguably represent a "higher-risk, higher-reward" cell source, a more systematic, methodical approach to preclinical optimization seems warranted and would help facilitate successful clinical translation.

First and foremost, the feasibility and efficacy of large-scale remuscularization must be firmly established in a large-animal model, and the specific dose and composition of the cardiomyocytes needs to be better defined. For example, doses of human ESC derivatives ranging from 2 million to 1 billion cells have been tested in large-animal models (Ye et al. 2014; Chong et al. 2014), suggesting that the field is far from a consensus in terms of what mechanism of action and cell numbers are most likely to mediate clinically relevant beneficial effects. The optimal cellular composition also needs to be firmly established. For example, stromal and endothelial cells make up the majority of the heart by nuclei (Bergmann et al. 2015), and so it makes intuitive sense that these cells should be incorporated in a heart

regeneration strategy. However, human ESC-derived multipotent cardiovascular progenitors did not outperform committed cardiomyocytes in a recent head-to-head study (Fernandes et al. 2015).

Another issue that needs further optimization in large-animal studies is the approach to cardiomyocyte delivery. The various methods of cardiomyocyte delivery described above (e.g., surgical injection or catheter-based injection of cell suspensions versus implantation of an engineered muscle “patch”) should be tested, ideally in a head-to-head comparison, and that approach resulting in the best retention of viable cells and/or greatest beneficial effects on LV contractile function should be prioritized in early clinical trials. To our knowledge, no one has reported the viability of PSC-CMs after passage through a cell-delivery catheter even in vitro, although this potential method of delivery has been frequently proposed. For any approach involving cell injection, large-animal studies are also required to inform the optimal number of injections, volume per injection site, and distribution of sites.

The risk of graft-related arrhythmias is a particularly important hurdle to successful translation for the reasons described above. Even if this risk can be greatly minimized by enhancing the input cell population (e.g., by employing more mature and/or ventricular-enriched PSC-CMs), additional preclinical studies seem warranted to determine the electrophysiological responses of graft tissue to antiarrhythmic drugs and other relevant conditions likely to be encountered in heart failure patients (e.g., ischemia). An informed decision will also have to be made as to whether all early PSC-CM recipients will be required to have an automatic implantable cardioverter-defibrillator (AICD), as was the case during clinical trials with skeletal myoblasts. Such a requirement would obviously provide a greater margin of safety and so may be entirely appropriate, but it would necessarily affect economic considerations and target patient population during trial design.

Finally, immunosuppression is another important issue and one that is particularly challenging to investigate based on xenotransplantation experiments involving human cardiomyocytes implanted into animal models. Allotransplantation experiments within a single species would likely better model responses to cardiomyocyte transplantation in humans. For example, monkey ESC-CMs could be transplanted into allogeneic monkeys and evaluated for the minimal level of immunosuppression needed to ensure graft survival.

9.11 What Is the Status of PSC-Derived Cardiomyocyte Transplantation in Human Trials?

To date there have been no clinical trials testing the transplantation of committed cardiomyocytes from PSCs or any potential source. However, investigators in France recently initiated a clinical trial to test human ESC-derived cardiovascular progenitor cells delivered via surgery in a fibrin scaffold, although their objective is not to achieve direct remuscularization. The Transplantation of Human Embryonic Stem Cell-Derived Progenitors in Severe Heart Failure (ESCORT, NCT02057900) trial is a phase 1 clinical trial that aims to recruit six patients undergoing cardiac surgery (e.g., coronary artery bypass grafting or mitral valve

repair/replacement) to simultaneously receive an epicardial implant of multipotent cardiovascular progenitor cells. These cells are generated by treating undifferentiated human ESCs with the growth factor BMP2 and SU-5402, a small-molecule fibroblast growth factor receptor (FGF)-specific tyrosine kinase inhibitor. Somewhat paradoxically, these cells are defined by their expression of the surface marker SSEA-1, which is expressed by multipotent cardiovascular progenitors but is more commonly used as a pluripotency marker (Menasche et al. 2015a). A relatively small number of cells (up to 10 million) will be implanted in the ESCORT trial, with the expectation that cell implants will mediate beneficial effects via an indirect, paracrine mechanism of action rather than direct revascularization (Menasche et al. 2015a). In addition to the aforementioned requirement that the enrolled patients must already be undergoing an open-chest procedure, other inclusion criteria include: (1) age 18–81 years, (2) LV ejection fraction $\leq 35\%$, (3) MI older than 6 months, (4) New York Heart Association class III or IV heart failure despite optimal medical care, (5) AICD placement with cardiac resynchronization therapy as indicated, and (6) not eligible for heart transplantation.

In 2015, the ESCORT team released a case report describing their first enrolled patient (Menasche et al. 2015b). The patient is a 68-year-old, insulin-dependent diabetic woman with New York Heart Association class III heart failure from an anterolateral MI with a LV ejection fraction of 26% on maximal medical therapy. She underwent coronary artery bypass grafting with left internal thoracic artery (left internal mammary artery) to the left anterior descending artery without suitable targets for revascularization of the left circumflex coronary arterial tree. The progenitor cell-containing patch made with 4 million cells was grafted onto the lateral LV in the region visually identified to have scar. She received immunosuppression with methylprednisolone, cyclosporine, and mycophenolate mofetil. At 3 months she has not had any adverse events and her LV ejection fraction improved to 36% by echocardiographic evaluation with the akinetic lateral wall improving to moderately hypokinetic. Her improvement in LV ejection fraction is in line with the effects of coronary artery bypass grafting for ischemic cardiomyopathy in the setting of anterior wall viability (Sharma et al. 2011); however, whether the functional improvement of the lateral wall was due to improved collateral flow or the overlying cellular patch is unknown.

9.12 How Should Future Clinical Trials with PSC-Derived Cardiomyocytes Be Designed?

Cardiomyocyte transplantation has the potential for both tremendous benefit and serious risks that will vary depending on the patient population selected. It will be important to recognize and account for these differences in planning the first clinical trials of human PSC-CM transplantation. In our opinion, the first patient population to be considered for study should have severe, symptomatic (NYHA class III or IV) ischemic cardiomyopathy, because patients with less severe cardiomyopathy are usually well managed with contemporary medical therapy alone. In our view, the

most appropriate participants of such a trial can be further divided into four broad categories of patients: (1) individuals with another indication for cardiac surgery, (2) candidates for long-term mechanical circulatory support with a LV assist device (LVAD) with or without a subsequent heart transplant, (3) chronic heart failure patients who are not candidates for LVAD or heart transplantation, or (4) individuals with cardiogenic shock from acute MI. Each of these patient profiles has unique clinical characteristics that would affect trial design and outcomes.

Patients who are already undergoing cardiac surgery, as in the ESCORT trial, would allow for delivery of cardiomyocytes via transepical injection under direct visualization. As discussed above, this is the most studied delivery method to date and is arguably the approach by which the delivery of cardiomyocytes could be best controlled. Furthermore, since these patients will already have a cardiac surgery, the decision to transplant cardiomyocytes does not impose an additional sternotomy or surgery.

Another potential population that has been proposed for early clinical trials testing cardiomyocyte transplantation are end-stage heart failure patients receiving a LVAD. In this instance, the patients at question are obviously already undergoing an open-chest procedure (device implantation), and they will be receiving a device that is capable of taking over LV cardiac output, providing an additional margin of safety. That said, the support of an LVAD does not eliminate all risk; for example, a left-sided pump cannot compensate for right ventricular arrhythmias or right-sided failure. Importantly, the LVAD recipient population is further subdivided into two categories of patients: those being “bridged to transplantation” (i.e., supported with the device until a suitable donor heart becomes available) and those receiving the device as “destination therapy” (i.e., supported with the device permanently, as they are not candidates for heart transplantation). Each group has its pros and cons as potential subjects in a clinical trial of PSC-based cardiac therapy. Obviously, “bridge-to-transplant” patients have a “backup” plan in place, since they would be listed for heart transplantation. In this case, the engrafted heart would be removed at the time of transplantation, which would increase the margin of safety with regard to tumorigenesis while also providing invaluable information as to the structural fate of the graft cardiomyocytes. At the same time, a major disadvantage of “bridge-to-transplant” patients is that graft cells could allosensitize them, negatively affecting their transplant candidacy or outcomes post-transplant. A major drawback with both “bridge-to-transplant” and “destination therapy” patients is that human PSC-CM recipients would likely require chronic immunosuppression to avoid graft cell rejection, thereby greatly increasing their risk of serious LVAD or driveline infection. Finally, while the LVAD patient population has a number of safety advantages, it would be challenging to determine efficacy given the coexisting device, and there are obvious ethical issues inherent in cell transplantation in individuals that are unlikely to be benefit (and may be harmed).

On the other hand, end-stage heart failure patients who are not candidates for LVAD or heart transplantation would have to accept all of the risks of cardiomyocyte transplantation and immunosuppression without any “backup” strategy in the case of a bad outcome (e.g., graft-related tumor). Looking beyond early safety

studies, an important consideration here would be whether such individuals might stand any chance of benefiting from cardiomyocyte transplantation. Given that human PSC-CMs have not mediated significant beneficial effects in chronic MI in the limited number of animal studies to date, it is possible that these patients would be accepting all of the risks with little or no likelihood of benefit.

By contrast, animal studies have so far suggested that human PSC-CM transplantation can have a significant beneficial effect in the acute phase post-MI. The risk-benefit ratio calculation here is very challenging, because most patients respond well to medical therapy following acute MI and go on to have only mild or moderate ischemic cardiomyopathy. Because it is not straightforward to identify and enroll those acute MI patients that will go on to have more serious dysfunction, one would likely need to have a large body of preclinical (and ideally clinical) safety data before contemplating cardiomyocyte transplantation in this context. One could conceivably target acute MI patients with cardiogenic shock that are anticipated to need long-term circulatory support (e.g., LVAD), but this is a relatively small proportion of patients and would make recruitment very challenging.

In summary, we think that currently there is a division between the clinical scenario where human PSC-CM transplantation will most likely be beneficial (e.g., acute MI) and the patient population with the greatest clinical need. Since patients with severe, chronic ischemic cardiomyopathy comprise the most likely target population for human PSC-CM transplantation, it seems important that the preclinical data demonstrate benefit in this disease context. As such, clinical benefit needs to be shown in small- and large-animal studies that best model patients with chronic ischemic cardiomyopathy. Once the efficacy of human PSC-CM transplantation is firmly established in chronic ischemic cardiomyopathy, then planning could begin for human clinical trials keeping in mind the different patient groups discussed above.

Conclusions

Cardiomyocyte transplantation is not yet ready to be tested in human clinical trials, but tremendous progress has been made in recent years through *in vitro* work and increasingly relevant animal models of MI. Cardiomyocyte manufacturing is now possible at a scale and cardiac purity that would have seemed impossible just a few years ago. Recent preclinical studies have demonstrated the feasibility of remuscularizing a significant fraction of the infarct scar with electrically integrated new myocardium, and impressive beneficial effects on LV contractile function have been reported, at least in small-animal MI models. Going forward, the field must address a number of practical uncertainties including the most appropriate dosage, cell composition, route of administration, and immunosuppressive therapy. These are issues that should be addressed first in large-animal studies, which are costly, tedious, and absolutely essential. The field also needs to demonstrate functional efficacy following cardiomyocyte transplantation in a more clinically relevant large-animal model and must satisfactorily overcome concerns about graft cell survival, arrhythmias, and tumor formation. Post-MI heart failure is a disease with high morbidity and mortality, so the need for new

therapeutic options is acute. Cardiomyocyte transplantation is a highly promising approach, but its effective translation will require patience, hard work, cooperation, and a methodical approach in preclinical and first-in-human studies.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants performed by any of the authors. Animal research was performed in accordance with institutional, local, and federal regulations. This work was supported by T32 HL007312 (MEH), Future Leader Fellowship (ID 100463) from the National Heart Foundation of Australia (JJHC) and Sydney Medical School Foundation Fellowship (JJHC), RO1 NIH HL117991 (MAL), and funding from the McEwen Centre for Regenerative Medicine, the Toronto General Hospital Research Institute, the Ontario Institute for Regenerative Medicine, and the Peter Munk Cardiac Centre (MAL).

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