

## Translational aspects of cardiac cell therapy

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Received: April 15, 2015; Accepted: May 13, 2015

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### Abstract

Cell therapy has been intensely studied for over a decade as a potential treatment for ischaemic heart disease. While initial trials using skeletal myoblasts, bone marrow cells and peripheral blood stem cells showed promise in improving cardiac function, benefits were found to be short-lived likely related to limited survival and engraftment of the delivered cells. The discovery of putative cardiac 'progenitor' cells as well as the creation of induced pluripotent stem cells has led to the delivery of cells potentially capable of electromechanical integration into existing tissue. An alternative strategy involving either direct reprogramming of endogenous cardiac fibroblasts or stimulation of resident cardiomyocytes to regenerate new myocytes can potentially overcome the limitations of exogenous cell delivery. Complimentary approaches utilizing combination cell therapy and bioengineering techniques may be necessary to provide the proper milieu for clinically significant regeneration. Clinical trials employing bone marrow cells, mesenchymal stem cells and cardiac progenitor cells have demonstrated safety of catheter based cell delivery, with suggestion of limited improvement in ventricular function and reduction in infarct size. Ongoing trials are investigating potential benefits to outcome such as morbidity and mortality. These and future trials will clarify the optimal cell types and delivery conditions for therapeutic effect.

**Keywords:** cell therapy • cardiac progenitor cell • cardiac regeneration • direct reprogramming • combination cell therapy • biomaterials

### Introduction

Ischaemic heart disease, in the form of acute myocardial infarction (MI) and resultant ischaemic cardiomyopathy, remains the leading cause of morbidity and mortality worldwide [1]. Despite significant improvements in cardiac care over the past 50 years especially in primary and secondary prevention, approximately 1 million MIs still occur each year in the United States. Many of these patients go on to develop heart failure, which now affects over 5 million patients [2]. While medications such as beta-blockers, angiotensin-converting enzyme inhibitors, and aldosterone

antagonists can ameliorate decline in heart function, end-stage heart failure frequently necessitates complete or partial replacement of cardiac function with either heart transplant or a mechanical assist device [3].

With a MI, the heart can lose over a billion cells, approximately 25% of its mass [4]. To compensate for the loss of cells, the affected area forms fibrotic scar tissue by activated fibroblasts and the immune response. Although tissue regeneration is a phenomenon occurring in adult mammalian tissues such as liver, skeletal muscle, bone and skin, the ability of the adult heart to renew itself is limited [5]. This is not the case for lower vertebrates that are able to fully regenerate cardiac tissue following substantial injury [6]. Until recently, the heart itself was thought to be a terminally

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differentiated organ. Bergmann *et al.* utilized the increased global levels of  $^{14}\text{C}$  from Cold War atomic bomb testing to date cardiomyocytes in patients who lived during that time period, and found evidence based on mathematical modelling for renewal of cardiomyocytes of up to 1% per year for a 20 year old [7]. This renewal rate gradually decreased with age, to a yearly rate of 0.4% by age 75. Through these and other studies [8–10], it is now understood that endogenous repair mechanisms do exist in the adult mammalian heart, albeit at a capacity which is unable to fully counteract the damage caused by a MI.

Cardiac cell therapy, either through transplantation of exogenous cells or stimulation of endogenous resident cells, has been widely studied as a potential method for repair and regeneration of cardiac tissue. This manuscript explores the translational aspects of cardiac cell therapy, including cell source selection for exogenous delivery, strategies to regenerate cardiac tissue through direct reprogramming of endogenous cells, and enhancement of native cardiomyocyte proliferation through delivery of growth and transcription factors. We will also explore future directions in the field including combination cell therapy and bioengineering techniques.

## Exogenous cell delivery

A wide variety of stem cell types have been evaluated for therapeutic delivery for cardiac repair, ranging from unipotent skeletal myoblasts to pluripotent embryonic stem cells (Figure 1). Starting with early studies utilizing skeletal myoblasts and bone marrow stem cells, the rationale for stem cell delivery was predicated on the speculation that ‘plasticity’ of adult stem cells may lead to transdifferentiation of these cell types into cardiomyocytes to ‘regenerate’ native damaged tissue. Although it is now understood that the positive effects of cell delivery on cardiac function in these early studies may have resulted from a paracrine effect rather than true cell engraftment and differentiation into cardiomyocytes, multiple pre-clinical and clinical studies have been performed demonstrating relative safety and modest efficacy of these cell types. With the discovery of cardiac ‘progenitor’ cells as well as advancements in pluripotent stem cell (PSC) derivation, there is now the possibility for delivery of cardiomyocyte progenitors and cardiomyocytes capable of true engraftment and regeneration of cardiac tissue. Many questions remain with exogenous cell delivery techniques, including the choice of the best cell type for therapeutic effect as well as proper delivery method, given the low engraftment rates as well as the propensity for arrhythmogenesis.

### Skeletal myoblasts

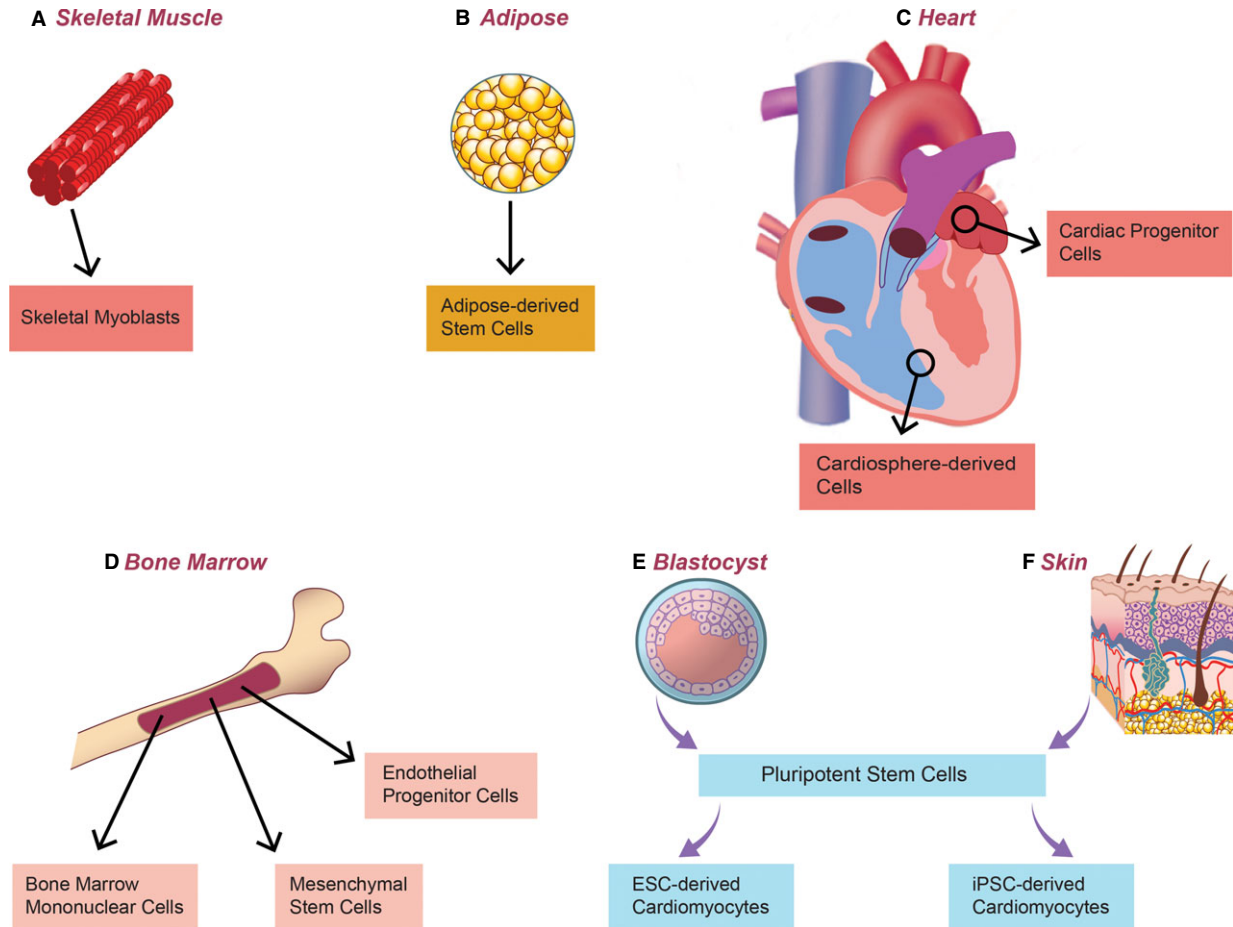
Initial studies using a cell-based strategy for ischaemic heart disease relied on skeletal myoblasts, based on its ability to regenerate skeletal muscle through proliferation of quiescent satellite cells located under the basal lamina [11]. Advantages of using this cell type include easy expansion *ex vivo*, and the ability to use an autologous source. Although preclinical studies demonstrated potential

for intramyocardial injection of skeletal myoblasts to improve LV function likely through a mechanical scaffolding effect [12, 13], multiple clinical trials including MAGIC [14] and MARVEL [15] have since revealed lack of efficacy when compared to placebo. Further studies showed that the injected cells do not integrate electromechanically with the surrounding myocardium (as they do not express connexin 43) [16], have a propensity to induce arrhythmias (especially dangerous ventricular tachyarrhythmias) [17], and do not regenerate myocardium [18]. Considering lack of significant clinical improvement and their potential arrhythmogenic hazards, skeletal myoblasts have fallen out of favour as a therapeutic candidate.

### Bone marrow cells

As a result of decades of experience in the haematological realm for bone marrow transplants, bone marrow cells have been closely examined as a therapeutic option for cardiac cell therapy (Table 1). These cells contain many inherent advantages, including ease in harvesting pure cell populations in large numbers, ability to be used allogeneically, and composition including fractions of stem and progenitor cells of different types. For these reasons, unselected bone marrow mononuclear cells have been the most widely tested in pre-clinical and clinical trials for cardiac therapy. Although an early study by Orlic *et al.* supported the idea that unselected bone marrow cells have the capability to differentiate into cardiomyocytes [8], this has since been discredited by a number of independent investigations [19, 20]. Several selected studies did, however, demonstrate improvement in cardiac function as well as decrease in infarct size [21, 22]. Other studies specifically examined the haematopoietic stem cell (HSC) subset of the unselected bone marrow population. Characterized by multiple distinct markers including CD133 and CD34 [23]. HSCs were shown in some pre-clinical studies to promote neovascularization and prevent LV remodelling [24]. This subset of cells (accounting for less than 0.1% of unfractionated bone marrow mononuclear cells) may partially account for the positive effects of unselected bone marrow cell therapy [25].

Closely related to HSCs is the subset of circulating bone marrow mononuclear cells that are thought to specifically differentiate into endothelial cells. Endothelial progenitor cells (EPCs) were first characterized in 1997 by Asahara *et al.* [26] as expressing the HSC marker CD34 as well as an endothelial marker protein (most commonly VEGF-R2), and are thought to play a major role in neovascularization and maintenance of endothelial integrity under conditions of myocardial ischaemia [27]. Initial animal studies using intramyocardial delivery of CD34<sup>+</sup> cells in both rat [28] and porcine [29] models of MI showed promising improvements in cardiac function. These results led to several clinical studies specifically investigating cardiac transplantation of autologous CD34<sup>+</sup> cells in chronic ischaemia (ACT34-CMI) [30], acute MI (TOPCARE-AMI) [31], and post-MI (TOPCARE-CHD) [32]. All demonstrating safety of the therapy with some evidence of efficacy. The ongoing RENEW study will examine the efficacy of intramyocardial autologous CD34<sup>+</sup> cell transplantation in patients with refractory angina [33].



**Fig. 1** Cell and tissue sources of cells for exogenous cell delivery. Multiple clinical trials have investigated non-cardiac cells including (A) skeletal myoblasts, (B) adipose-derived stem cells, and (D) bone marrow-derived stem cells, with limited evidence of cell engraftment or clinical efficacy. Clinical trials utilizing cells obtained from biopsied cardiac tissue (C) including cardiac ‘progenitor’ cells and cardiosphere-derived cells have provided the strongest evidence to date for clinical efficacy of exogenous cell therapy. Embryonic stem cells (E) and induced pluripotent stem cells (F) can be used as a source of cardiomyocytes potentially capable of electromechanical integration into native cardiac tissue.

More recently, it has been reported that there may be both ‘early’ and ‘late’ types of EPCs. Early EPCs are obtained from early 4–7 day *in vitro* cultures and express specific endothelial markers CD31 and TIE2, while late EPCs are cultured for at least 2–3 weeks *in vitro* and then express additional markers such as VE-cadherin and von Willebrand factor [34]. It still remains unclear whether a specific EPC subset may promote substantial neovascularization in the injured myocardium, or whether the distinction exists purely *in vitro*.

Besides exogenous transplantation of bone marrow cells, a related therapeutic strategy has been the use of haematopoietic growth factors including granulocyte colony-stimulating factor [35], granulocyte macrophage colony-stimulating factor [36] and macrophage colony-stimulating factor [37] in the setting of myocardial injury. Their beneficial effect is predicated on mobilization of endogenous bone marrow stem cells including HSCs and EPCs which may

then improve cardiac function through putative paracrine effects as well as a direct angiogenic effect on ischaemic tissue [38–40]. Initial pre-clinical murine studies utilizing these factors demonstrated reduced LV remodelling as well as improved cardiac function [37, 41, 42]. However, a number of pilot clinical trials have since shown variable outcome in terms of efficacy, with most of them unable to reproduce the favourable outcome seen in the animal studies [43–46].

A wide heterogeneity exists in the specific bone marrow cells used for the pre-clinical and clinical studies in this field, with differences in the cell isolation, storage and enrichment processes [47]. The wide clinical experience with bone marrow cells for cardiac therapy has had mixed results, likely because of this heterogeneity. Ongoing trials with specific populations of purified bone marrow cells as described above will shed light on the promise of this cell type for future cardiovascular therapy.

## Mesenchymal stem cells

Another source for allogeneic cell therapy consists of mesenchymal stem cells (MSCs), also known as mesenchymal stromal cells or colony forming unit-fibroblasts. These were first isolated from bone marrow stroma and described by Friedenstein *et al.* [48] more than 40 years ago, and have been shown in the intervening decades to be a multi-potent source of mesoderm (as well as some non-mesoderm) derived tissues including osteoblasts, chondrocytes, adipocytes, skeletal muscle, hepatocytes and even neurons [49, 50]. The ability of MSCs to differentiate into cardiomyocytes is somewhat in dispute, however, with some studies demonstrating transdifferentiation of MSCs to cardiomyocytes [51, 52] while many others showing very limited cardiomyogenic potential [53, 54]. Despite this controversy, MSCs have been eagerly pursued as a cell-based source for cardiac repair, because of their many other favourable properties including their immunomodulatory properties and their easy isolation and amplification from an allogeneic source [55].

The main role of the MSC is thought to be as a controller of stem cell niches, most importantly that of the HSCs in the bone marrow, but also in other tissues including the gut and hair follicles [56]. There is no uniform definition for MSCs, but the International Society of Cell Therapy has proposed criteria for MSCs including: (i) the ability to adhere to plastic under normal culture conditions and display a fibroblast-like morphology, (ii) the ability to differentiate into osteoblasts, adipocytes, and chondrocytes *in vitro* and (iii) expression of surface markers CD73, CD90 and CD105, in the absence of CD11b, CD14, CD19, CD34, CD45, CD79a and HLA-DR (Human leukocyte antigen) [57].

Mesenchymal stem cells produce their immunomodulatory effects through their unique immunophenotype, the secretion of soluble factors, and through interactions with both the innate and adaptive immune cells. As they are negative for MHC II (Major Histocompatibility Complex), B7, and CD40, MSCs are tolerated well when allogeneically transplanted. By secreting factors such as interleukin-6, transforming growth factor (TGF)- $\beta$ 1, and prostaglandin E2, MSCs suppress innate immune cell inflammatory responses such as the respiratory burst function of neutrophils [58] and production of INF- $\gamma$  (Interferon alpha) by natural killer cells [59]. In addition, MSCs have been shown to modulate the adaptive immune system as well, mainly through suppression of T cell proliferation [60]. It is thought that these properties may ameliorate ischaemic cardiac damage especially during the initial immune response to injury.

Mesenchymal stem cells have been isolated from many different tissue types including bone marrow, adipose tissue, lung tissue, umbilical cord blood and peripheral blood, but are most easily harvested from the bone marrow and adipose tissue. In particular, adipose-derived mesenchymal stem cells (ADCs) have the attractive feature of being easily harvested and isolated from an allogeneic source through liposuction with a high yield. Thus, most pre-clinical and clinical studies have focused on delivery of MSCs isolated from these two sources. Large-animal studies reported the ability of MSCs to decrease infarct size and improve ventricular function [61, 62]. These studies used multiple delivery methods including intravenous injection, intracoronary infusion, catheter-based intramyocardial

injection and direct surgical myocardial injection [49]. As with other cell types studied for cardiac repair, the exact mechanisms for the improvement in heart function are unclear, but are likely related to possible anti-inflammatory effects as well as paracrine signalling to recruit endogenous stem cells and promote healing by minimizing fibrosis.

Based on the promising initial pre-clinical results, multiple clinical trials have evaluated the use of bone marrow and ADCs both in acute cardiac ischaemia as well as ischaemic cardiomyopathy. Studies involving intravenous [63] intracoronary infusion [64] and intramyocardial injection [65] of bone marrow derived MSCs as well as intracoronary infusion of ADCs (APOLLO) [66] have demonstrated safety of autologous and allogeneic cells in acute and sub-acute MI, with modest improvement in LV ejection fraction. Early clinical trials using MSCs in ischaemic cardiomyopathy, most notably TAC-HFT (Transendocardial Autologous Mesenchymal Stem Cells and Mononuclear Bone Marrow Cells in Ischemic Heart Failure Trial) [67] comparing MSCs with BM mononuclear cells and POSEIDON [68] comparing allogeneic with autologous MSCs appear to confirm the safety of this cell type, although determination of clinical efficacy will necessitate larger trials. The PROMETHEUS study [69] utilizing autologous MSCs in patients with chronic ischaemic cardiomyopathy undergoing coronary artery bypass grafting (CABG) points to efficacy based on improvement in ventricular contractile function and decrease in scar size. Two clinical studies looking at safety and efficacy of adipose-derived MSCs have recently been completed for both acute ischaemia [195] and chronic ischaemic cardiomyopathy [196], and the results of these studies will delineate the potential regenerative efficacy of this particular cell type in cardiac repair.

## Cardiac progenitor cells

Until recently, it was thought that the heart was a fully differentiated organ without the capacity for regeneration. Multiple groups [9], including ours [10], have since found that post-natal generation of new cardiomyocytes does indeed occur, albeit at a very low rate. Many types of putative 'cardiac progenitor cells' (CPCs) have been reported, with the shared definition that they are clonal multi-potent cells capable of self-renewal and differentiation into the three major cardiac cell types. The most clinically relevant, of these types for cell therapy have been the c-kit<sup>+</sup> cell [70] and the cardiosphere-derived cell (CDC) [71], while Sca-1<sup>+</sup> cells [72], Isl-1<sup>+</sup> cells [73, 74], SSEA-1<sup>+</sup> cells [75, 76], side-population cells [77] and telocytes [78, 79] have also been the subject of research interest.

Cardiac progenitor cells with the ability to differentiate into cardiomyocytes, endothelial cells and smooth muscle cells were first reported in the rat heart by Beltrami *et al.* in 2003 [70], and later in the human heart [80]. These cells reportedly expressed the tyrosine kinase receptor c-kit (CD117), a marker of stemness, lacked hematopoietic lineage markers, and were found to be multi-potent, clonal and self-renewing [81]. Early studies utilizing human c-kit<sup>+</sup> CPCs in an infarction model of immunodeficient mice reported successful engraftment, differentiation into the three major cardiac cell types,

**Table 1** Notable cardiac cell therapy clinical trials recently completed or currently in progress

Study	Phase	Cell type	Population	Primary outcome(s)	Status/estimated completion
ALLSTAR [191]	I/II	Allogeneic CDCs	Recent myocardial infarction with resultant ischaemic cardiomyopathy	Reduction in infarct size assessed by MRI	Recruiting/October 2015
RENEW [192]	III	Autologous CD34 <sup>+</sup> EPCs	Chronic myocardial ischaemia	Improvement in total exercise time on Modified Bruce Protocol	Active, not recruiting/June 2016
Pre-SERVE-AMI [193]	II	Autologous CD34 <sup>+</sup> HSCs	Acute ST-elevation myocardial infarction	Safety (measured by adverse events) and efficacy (measured by improvement in SPECT MPI)	Active, not recruiting/December 2016
AMICI [194]	II	Allogeneic HSCs	Acute myocardial infarction	Safety (defined by major adverse cardiac and cerebral events)	Recruiting/June 2018
ADVANCE [195]	II	Autologous ADCs	Acute ST-elevation myocardial infarction	Reduction in infarct size assessed by MRI	Completed
MyStromalCell [196]	II	Autologous ADCs	Chronic ischaemic cardiomyopathy	Improvement in exercise test	Completed
ATHENA [197]	II	Autologous ADCs	Chronic myocardial ischaemia	Safety (adverse events) and feasibility (change in mVO <sub>2</sub> , LVESV/LVEDV, EF and symptoms)	Active, not recruiting/May 2019
ATHENA II [198]	II	Autologous ADCs	Chronic myocardial ischaemia	Improvement in symptoms (assessed by Minnesota Living with Heart Failure Questionnaire)	Active, not recruiting/May 2019
STEMI [199]	II	Allogeneic MSCs	Acute ST-elevation myocardial infarction	Safety (by major adverse cardiac events)	Active, not recruiting/May 2017
STEM-104-M-CHF [200]	II	Allogeneic MSCs	Chronic non-ischaemic cardiomyopathy	Safety	Recruiting/May 2015
Prochymal [201]	II	Allogeneic MSCs	Acute myocardial infarction	Improvement in left ventricular end systolic volume	Active, not recruiting/February 2016

CDCs: cardiosphere-derived cells; EPCs: endothelial progenitor cells; HSCs: hematopoietic stem cells; ADCs: adipose-derived stem cells; MSCs: mesenchymal stem cells; MRI: magnetic resonance imaging; SPECT MPI: single photon emission computed tomography myocardial perfusion imaging; mVO<sub>2</sub>: mixed venous oxygen saturation; LVESV: left ventricular end systolic volume; LVEDV: left ventricular end diastolic volume; EF: ejection fraction.

and improvement in cardiac function by echocardiography [80]. In a porcine chronic infarct model, c-kit<sup>+</sup> CPCs were first isolated and expanded from right atrial appendage resections, and then delivered *via* coronary artery infusion through a catheter [82]. Results showed successful engraftment of delivered cells and improvement in LV function, setting the stage for translation into human clinical trials. The SCIPIO [83] trial utilized autologous c-kit<sup>+</sup> CPCs harvested and expanded from the right atrial appendage at the time of CABG, with intracoronary infusion at a mean of 113 days after CABG. At 1 year after infusion, LV function by echocardiography was found to increase by 12.3% ± 2.1% compared to the control group, while the infarct size by magnetic resonance imaging (MRI) was found to decrease significantly.

Another CPC type under intense investigation has been the CDC [84]. First isolated from mice and human biopsy samples in 2004 [71] and later in dogs [85, 86], these cells were expanded using spheroid culture technique. These cells were then found to form aggregates of a heterogeneous cell population that expressed stem cell markers such as c-kit, Sca-1 and CD34. Further characterization revealed multi-potentiality and clonogenicity of the cells, with cells at varying stages of differentiation (based on expression of cardiac lineage markers such as cardiac Troponin-I, atrial natriuretic peptide and CD31) depending on their location within the cell mass. The cells in the core were found to be mainly proliferating c-kit<sup>+</sup> cells, with more differentiated cells as well as MSCs (characterized by expression of CD90 and CD105) towards the periphery, potentially indicating a role for MSCs in promoting CPC differentiation and renewal. The mediator for CDC-induced regeneration may be related to exosome delivery of miR-146a [87, 88]. More recently, it was found that THY-1 (Thymocyte antigen 1) (CD90) receptor expression could also be used to delineate CDCs with divergent cardiac differentiation potential into either mesenchymal/myofibroblast cells or cardiomyocytes [89]. Initial pre-clinical studies involving injection of CDCs in an immunodeficient murine infarction model showed improvement in echocardiographic cardiac function [90]. This led to a porcine study [91] using intracoronary delivery of CDCs which demonstrated reduction in relative infarct size by MRI. Soon thereafter, the initial human clinical trial (CADUCEUS) [92] studying autologous CDCs obtained through endomyocardial biopsy reported decreased scar size by MRI in patients receiving intracoronary infusion of CDCs after AMI.

The demonstration of clinical safety in both SCIPIO and CADUCEUS (along with suggestion of efficacy) has been encouraging for the field, but efficacy will have to be confirmed after longer time periods and through larger clinical trials involving sample sizes powered for such a determination. The ALLSTAR trial [191] investigating the delivery of allogeneic CDCs in patients with LV dysfunction after MI will shed more light on the future of this cell type as a therapeutic option.

## Pluripotent stem cells

Pluripotent stem cells have the ability to differentiate into all cell lineages, and hence offer novel treatment options for many intractable diseases including end-stage heart failure. Human embryonic stem

cells (hESCs) have been investigated as a source of cells for cardiac repair through *ex vivo* differentiation into either cardiac 'progenitors' [76] or into mature cardiomyocytes [93]. However, limitations include the inability to isolate pure tissue-specific progenitors capable of robust engraftment and regeneration, potential risk of teratoma formation from residual PSCs in the transplanted cells [94], and ethical concerns with their generation.[95] In addition, it is uncertain that hESCs can functionally engraft and electromechanically couple into the surrounding myocardium. These concerns have limited the clinical translation of hESCs for cardiac therapy.

The report by Yamanaka in 2006 [96] that terminally differentiated murine fibroblasts could be 'reprogrammed' to a primitive embryonic stem cell-like state through introduction of four specific transcription factors (Oct3/4, Sox2, c-Myc and Klf4) brought new hope to cardiac regenerative medicine. These cells, called induced PSCs (iPSCs), may bypass the ethical concerns associated with ESCs, and serve as a potentially unlimited source of cells for transplantation. While murine studies reported engraftment of iPSCs into infarcted myocardium [97], concerns for tumourigenicity have greatly limited further investigation using direct transplantation of iPSCs.

The most promising application of PSCs in cardiac regenerative medicine has been their use as a cell source for derivation of adult cardiomyocytes for transplantation. While early protocols for differentiating ESCs into cardiomyocytes generated less than 1% yields [93], more recent differentiation protocols have achieved yields of up to 70% [98]. Further enrichment for ESC-derived cardiomyocytes can be accomplished through use of a cardiac-specific promoter for expression of a fluorescent protein [99], sorting for cell surface markers [100–102] or sorting *via* Raman spectroscopy [103]. Our group has reported on hESC-derived ROR2(+)/CD13(+)/KDR(+)/PDGFR $\alpha$ (+) cells that give rise to cardiomyocytes [104] as well as endothelial cells and vascular smooth muscle cells. To date, ESCs, iPSCs [105] and even parthenogenetic PSCs [106] have been successfully differentiated into cardiomyocytes. Investigation into the electrical-mechanical properties of derived cardiomyocytes have found them to exhibit significant automaticity with immature action potential [107] and contractile [108] properties, highlighting the need for further development of differentiation conditions capable of producing cardiomyocytes of more mature phenotype compatible with native myocardium.

*In vivo* studies utilizing PSC-derived cardiomyocytes have been promising, with early rodent studies in acute [93] and chronic [109] infarct models demonstrating improvement in ventricular contractile function. More recently, hESC-derived cardiomyocytes have been shown in a primate model of ischaemia-reperfusion injury to engraft into infarcted host tissue, 'remuscularize' the infarct region, and electromechanically couple to surrounding host cardiomyocytes [110]. However, the presence of arrhythmias were reported in all animals receiving cell therapy, highlighting the potential problem with the arrhythmogenicity of transplanted cell. Whether these cells are inherently arrhythmogenic or serve as a nidus to induce arrhythmias is still not entirely clear. Future translation of this approach will require further understanding to eliminate the arrhythmogenicity inherent in transplanted cardiomyocytes before human clinical studies can be initiated.

## Endogenous cell therapy

Cell therapy relying on exogenous delivery of cells has provided great promise for the treatment of cardiovascular diseases. However, issues such as low cell survival, poor engraftment and limited functional maturation have emphasized the need to develop novel therapeutic alternatives. Regeneration of cardiac tissue through use of endogenous cardiac cells, as with direct reprogramming of resident cardiac fibroblasts or stimulation of native cardiomyocyte proliferation, can potentially sidestep the inherent limitations of exogenous cell delivery.

### Direct reprogramming of endogenous cells

Shortly after Yamanaka's report of reprogramming of somatic cells to iPSCs [96], the ability of these cells to differentiate into functional cardiomyocytes was readily demonstrated [105]. However, as with ESCs, the utilization of iPSC-derived cardiomyocytes raised a number of concerns such as potential differentiation towards alternative cell fates and teratoma formation once introduced to the heart. Direct reprogramming of fibroblasts to cardiomyocytes bypassing the pluripotent state was proposed as a method overcoming such hurdles [111, 112]. The abundance of fibroblasts in the heart [113] as well as their role following injury highlight the therapeutic potential of this approach. Direct conversion of fibroblasts into cardiomyocytes was first reported by Ieda *et al.* [112]. The authors showed that the combination of three transcription factors, GATA4, MEF2C and TBX5 (referred to as GMT) was able to convert mouse dermal and cardiac fibroblasts into cardiomyocyte-like cells, termed induced cardiomyocytes (iCMs). iCMs exhibited a gene expression profile similar to native cardiomyocytes while the fibroblast gene program was silenced, and a small fraction was able to spontaneously contract. However, the efficiency of the conversion was very low and the majority of iCMs was only partially reprogrammed. Similarly, Protze *et al.* demonstrated time-dependent conversion of mouse embryonic fibroblasts into cardiomyocyte-like cells through lentiviral expression of myocardin, MEF2C and TBX5 [114], while Song *et al.* reported the requirement of four factors GATA4, HAND2, MEF2C and TBX5 (GHMT) [115]. Although in the setting of the experiments by Ieda *et al.* [112], miRNAs were not required for reprogramming, Jayawardena *et al.* demonstrated that a panel of four miRNAs (miR-1, miR-133, miR-208 and miR-499) and a JAK inhibitor were sufficient for direct conversion of mouse fibroblasts into cells with characteristics of cardiomyocytes [116]. Muraoka *et al.* demonstrated that addition of miR-133 to the GMT combination resulted in a sevenfold increase in the generation of beating cardiomyocytes [117]. More recently, Addis *et al.* utilized a reporter system carrying the calcium indicator GCaMP under a Troponin-T promoter, and found that the combination of Hand2, Nkx2.5, Gata4, Mef2c and Tbx5 (HNGMT) was the most efficient in reprogramming of embryonic and adult mouse fibroblasts into functional cardiomyocytes [118]. It was then shown that small molecule inhibition of TGF- $\beta$  using SB432542 in combination with HNGMT further increased reprogramming efficiency [119]. Besides the specific combination of factors, the stoichiometric expression of

GMT factors significantly affects the efficiency of reprogramming and the quality of the iCMs [120]. In an effort to develop clinically applicable strategies for direct reprogramming, Wang *et al.* identified a cocktail of small molecules that was sufficient to reprogram mouse fibroblasts to ventricular-like cardiomyocytes in the presence of only one transcription factor, Oct4 [121].

However, the need for the development of regenerative strategies that do not require cell transplantation, as well as the low efficiency of direct reprogramming *in vitro* moved the field towards *in vivo* conversion of fibroblasts to cardiomyocyte-like cells [115, 116, 122, 123]. Retrovirus-mediated intramyocardial delivery of the GMT [122] or GHMT [115] combinations of transcription factors following MI resulted in successful direct reprogramming of fibroblasts into cardiomyocytes. The fraction of iCMs exhibiting characteristics of endogenous cardiomyocytes was significantly increased in the *in vivo* setting compared to *in vitro* reprogramming. Importantly the authors reported a decrease in infarct size and improvement in heart function [115, 122]. More recently, it was found that lentiviral-mediated administration of miR-1, miR-133, miR-208 and miR-499 into infarcted mouse hearts resulted in direct reprogramming of resident fibroblasts into cells with cardiomyocyte morphology and function, resulting in decreased infarct size and improved cardiac function [124].

Consistent with the findings in mice, recent studies have demonstrated the conversion of human fibroblasts to cells with cardiomyocyte characteristics [125–127]. Although human cells have been proven to be more challenging, various combinations of transcription factors and miRNAs (GATA4, HAND2, TBX5, myocardin and the miRNAs miR-1 and miR-133 [125], GMT, together with Myocardin, ZFPM2 (Zinc finger protein multitype 2) and TGF- $\beta$  [126], and GMT in addition to Mesp1 and Myocd) [127] have successfully produced human cardiomyocyte-like cells. Further studies in larger animals are required to explore their *in vivo* reprogramming potential.

### Activation of endogenous cardiomyocytes

Mammalian cardiomyocytes have long been considered as post-mitotic, terminally differentiated cells unable to re-enter the cell cycle. A number of studies have challenged this dogma, providing evidence of cardiomyocyte division in the adult heart [7, 9]. However, although the neonatal heart exhibits a robust regenerative capacity following injury [128, 129], in adults, the rate of cardiomyocyte proliferation is low and inadequate to replenish the lost tissue. In an effort to 're-activate' mature cardiomyocytes, a number of studies have suggested a variety of molecules ranging from growth and transcription factors, to cell cycle genes, to miRNAs, as potential therapeutic means to promote endogenous cardiomyocyte proliferation [130].

Administration of periostin, an extracellular matrix (ECM) protein produced by fibroblasts, has been shown to improve cardiac function and decrease infarct size following MI [131–133]. The beneficial effects of periostin have been attributed to increased cardiomyocyte DNA synthesis, mitosis and cytokinesis as well as increased angiogenesis [131–133]. However, the use of periostin as a therapeutic

agent remains controversial [133, 134], and further investigation is required.

Neuregulin is another protein that has exhibited strong therapeutic potential. This growth factor has been shown to promote cardiomyocyte cell cycle re-entry and cytokinesis in a mouse infarction model in addition to a pro-angiogenic and anti-apoptotic function [135]. Neuregulin treatment resulted in reduced scar size, ameliorated heart function and decreased hypertrophy [136]. More recently, Polizzotti *et al.* reported the existence of a 'therapeutic window', confined to the first postnatal days in mice and the first 6 months in humans, during which neuregulin treatment has remarkably higher efficiency in promoting cardiomyocyte regeneration [137]. Similarly, expression of the neuregulin co-receptor ERBB2 (human epidermal growth factor receptor 2) was shown to be sufficient for cardiomyocyte proliferation and tissue regeneration following injury [138]. Phase II clinical trials examining neuregulin administration as a therapeutic alternative for heart failure, have produced very promising results [139].

Insulin growth factor 1 (IGF1) and fibroblast growth factor 1 (FGF1) have also been proposed to promote cardiomyocyte proliferation [140–142]. Mice over-expressing IGF1 specifically in cardiomyocytes exhibited larger hearts as a result of cardiomyocyte hyperplasia [140], while in a recent report it was suggested that activation of the IGF1/Akt pathway coincides with a 'proliferative burst' of cardiomyocytes in preadolescent mice [141]. Likewise, Engel *et al.* demonstrated that a combination of FGF1 and a p38 inhibitor improved heart function and cardiomyocyte cycling in rats following MI [142]. Growth factor pathways such as IGF, Hedgehog and TGF- $\beta$  were also identified through an *in vivo* screening of cardiomyocyte proliferation modifiers [143].

In addition to the administration of exogenous proteins, alteration in the expression of transcription factors [144] as well as cell cycle genes [145, 146] represents an alternative therapeutic option. Namely, cardiomyocyte-specific deletion of a member of the TALE family of transcription factors (including Meis1) extended the proliferative window of postnatal cardiomyocytes from 7 to 14 days, while its overexpression reduced cardiomyocyte proliferation and decreased neonatal cardiac regeneration [144]. In the same context, Cheng *et al.* showed that constitutive myocardial expression of Cyclin A2 in mice resulted in enhanced cardiac function explained by cardiomyocyte cell cycle re-entry and increased regeneration [145]. More recently, adenovirus mediated delivery of Cyclin A2 in the peri-infarct area of pig hearts produced similar results [146].

Finally, several miRNAs involved in the regulation of cardiomyocyte proliferation have been proposed as potential therapeutic candidates [129, 147]. Porrello *et al.* elegantly demonstrated that inhibition of the miR-15 family results in cardiomyocyte proliferation and improved cardiac function following infarction in adult mice [129]. High-throughput screening of human miRNAs revealed forty miRNAs regulating cardiomyocyte DNA synthesis and cytokinesis *in vitro* while two of these (has-miR-590 and has-miR-199a) promoted cardiac regeneration and restored cardiac function in a mouse infarction model [147]. Similarly, the microRNA cluster miR-302-367 was shown to activate cardiomyocyte cell cycle re-entry and proliferation as well as decrease scar formation following MI in mice, partly because of inhibition of the organ size control

signalling pathway Hippo [148]. These recent data are in agreement with a number of studies which indicated that inactivation of the Hippo pathway promotes cardiomyocyte proliferation and cytokinesis after injury in both neonatal and adult mice [149], and that activation of the Hippo pathway effector protein Yap stimulates cardiomyocyte regeneration and improves cardiac function after injury in mice [150–152].

Development of novel therapies based on the delivery of molecules that are able to stimulate the endogenous cardiac cells to undergo proliferation offers significant advantages. Myocardial regeneration strategies, whether they involve fibroblast reprogramming or cardiomyocyte cell cycle re-entry would circumvent issues associated with more invasive cell-based therapies such as cell survival, engraftment and electromechanical coupling with resident cells.

## Future directions

As described in previous sections, the transplantation of several cell types has been shown in multiple pre-clinical and clinical studies to be a safe technique for potentially improving cardiac function, although evidence for true cardiac regeneration through successful engraftment of exogenous cells has been limited. This perhaps should not be surprising given the complexity of cardiac tissue. Multiple factors likely play a role in early cell death after intramyocardial delivery of exogenous cells, including the absence of necessary survival factors in the transplanted cells [153], loss of physiological signalling through interactions with the ECM [154], limited vascular supply in the local microenvironment [155], an inflammatory milieu in the aftermath of cell delivery [156] and inability to electromechanically couple with the host cardiomyocytes [16]. Strategies to improve cell retention and proliferation have thus focused on improving the immediate microenvironment into which the cells are delivered. One approach has investigated simultaneous delivery of pro-survival growth factors [93, 131, 157], of which the ideal combination has yet to be identified. Another technique being studied is the 'pre-conditioning' of cells into a pro-survival state through exposure to ischaemia, cytokines or heat shock [156].

Of particular interest are two approaches that seek to more closely recapitulate the particular microenvironment of a cardiac stem cell 'niche' to improve cell engraftment and survival. First, the co-delivery of two (or more) different types of cells takes advantage of potential synergistic and complimentary interactions between different cell populations. Second, bioengineering approaches such as the seeding and delivery of tissue engineered scaffolds could potentially enhance survival of delivered cells by providing the microstructural framework and extracellular cues necessary for cell viability.

## Combination cell therapy

The 'niche' model of adult stem cell self-renewal and differentiation, originally developed by Schofield in 1978 [158], describes a local microenvironment in which tissue (including cardiac tissue) is generated, maintained and repaired by stem cells under the regulation of a



complex interaction between the stem cells and surrounding niche support cells, soluble signalling molecules, and interactions with the ECM [159, 160]. As a large percentage of heart volume is comprised of interstitial tissue, it is thought that cardiac niches within the interstitial compartments of the myocardium and epicardium are responsible for potential cardiac regeneration. In particular, MSCs have been found to be involved in regulation of the cardiac as well as HSC niches [161]. Combination cell therapy builds upon this framework by co-delivering stromal support cells with stem cells to enhance cell survival and engraftment into the surrounding tissue. An early demonstration of this concept involved co-transplantation of satellite cells and MSCs in a murine model of Chagas cardiomyopathy, a combination which was found to improve cardiac function compared to control [162]. In another study, the combination of EPCs and MSCs was found to synergistically form functional vascular networks in Matrigel that remained patent at 4 weeks *in vivo* [163].

Another stromal support cell called the telocyte has been found to closely relate to CPCs at the level of the stem cell niche [164, 165] by directing progenitor cell differentiation *via* microRNA vesicular transfer [166, 167], making it another potential cell type for combination therapy. Separate from cardiac fibroblasts [168], these cells have been studied to improve cardiac function in rat models of MI [169, 170].

More recently, it has been found that MSCs induce proliferation and differentiation of c-kit<sup>+</sup> CPCs *via* interactions through connexin-43 gap junctions [161]. Based on this understanding, a combination approach using both MSCs and c-kit<sup>+</sup> CPCs was found to be synergistic in reducing scar size and improving cardiac function in a porcine model of MI when compared to either cell type alone [171]. A clinical trial to further evaluate this approach (AIRMID) is currently in the planning stage, and may further advance this field.

Another potential approach combines c-kit<sup>+</sup> CPCs with pericytes, a support cell thought to play an important role in vascular growth and angiogenesis through paracrine mediators [172]. An early-stage murine study demonstrated improved cardiac contractility by echocardiography as well as improved vascular proliferation and arteriogenesis [173], but further study will be required before such an approach can be translated to clinical trials.

## Bioengineering approaches

Normal functioning myocardium relies on a complex and dynamic interaction between multiple cell types, the ECM, and soluble signalling factors. In particular, an adult CPC 'niche' is governed by diverse interactions between surface-bound integrins (such as  $\alpha 1\beta 1$ ,  $\alpha 2\beta 1$ ,  $\alpha 10\beta 1$ ,  $\alpha 11\beta 1$  integrins) [174] and the ECM proteins collagen, elastin, laminin and fibronectin [175, 176]. The low rates of cell survival and engraftment in exogenous cell therapy is thus likely related in part to the dearth of these important physiological cues necessary for homeostasis immediately during and after delivery [177].

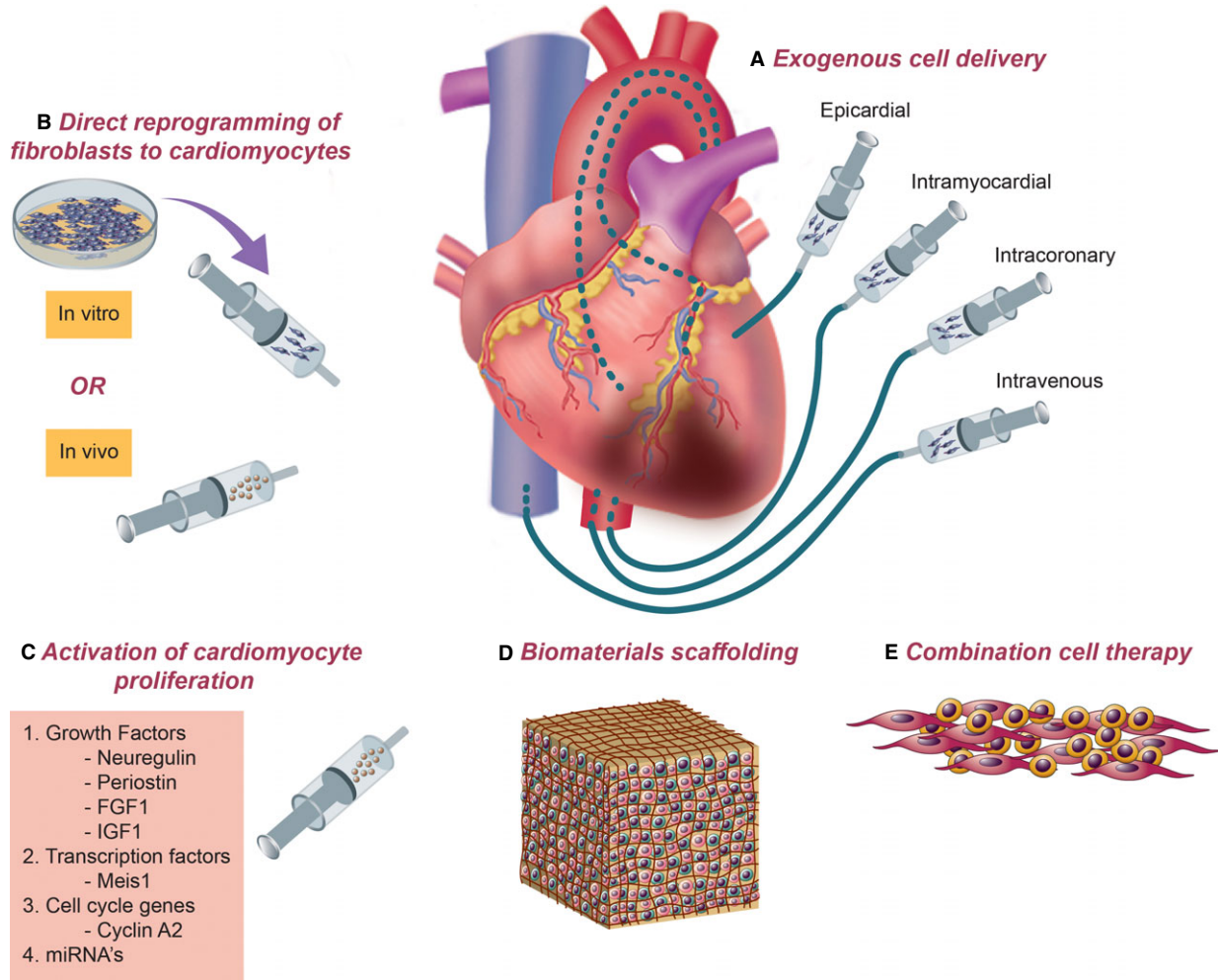
The ideal biomaterial complement to cell therapy should provide a proper three-dimensional structure with appropriate biological, bioelectrical, biomechanical and biochemical features specific for the cell type [178, 179]. Much attention has been focused on the incorpora-

tion of signalling molecules to influence cell biology. Strategies to date have ranged from co-delivery of ECM components such as collagen [180], Matrigel [181], fibrinogen [182], and de-cellularized ECM [183, 184], to non-ECM biological materials such as chitosan [185], to *in vitro* construction of seeded tissue-engineered scaffolds transplanted as cardiac patches [186]. Synthetic materials can be designed for specific properties; poly(lactic-co-glycolic acid) microcarriers can release growth factors in concert with co-delivered cells [187], and self-assembling peptide nanofibers can be co-delivered with cells to improve cell retention, direct differentiation and deliver protein [188–190]. However, further study regarding materials biocompatibility and biodegradation will be required prior to further clinical translation of this technology. Future efforts to develop resorbable, electrically conductive and biologically active materials with minimal modulus mismatch and adequate immunomodulatory properties would significantly advance this field. In addition, advances in tools and technologies to promote targeted delivery of progenitor cells to ischaemic and infarcted tissues as well as improvements in non-invasive cell tracking will reveal new insights on cell survival and integration.

## Conclusions

Cell-based therapy for amelioration and regeneration of cardiac tissue has been widely studied as a novel approach for the treatment of ischaemic heart disease. Multiple cell types have been intensely characterized and investigated as potential candidates for exogenous delivery. Initial studies using skeletal myoblasts, while encouraging in animal models, highlighted the inherent arrhythmogenic potential of exogenous cells that do not integrate electrically with the surrounding myocardium. Bone marrow cells, in both unselected and purified forms, have been under wide clinical investigation despite inconsistent outcomes. The unique immunomodulatory properties of MSCs may make them excellent candidates for combined therapy with another cell type. Pluripotent stem cells have emerged as an almost limitless source for derivation of differentiated cardiomyocytes with the potential to physiologically integrate with host myocardium both electrically and mechanically. Perhaps the cell types with some potential for cardiac repair have been the c-kit<sup>+</sup> and cardiosphere-derived CPCs, although independent large clinical trials are needed to confirm the preliminary results.

Despite these advances, significant obstacles remain in the field; low cell survival, poor engraftment and limited functional maturation (of progenitor cells) have blunted potential therapeutic benefit. While from a putative standpoint one would expect cell therapy to exert its beneficial effect by repopulating the damaged myocardium by the exogenous cells, others have argued that the delivery of exogenous cells may lead to recruitment of intrinsic cells capable of regenerating the damaged muscle; hence the loss of transplanted cells after a short time does not preclude the promise of stem cell therapy. Cell therapy strategies involving direct reprogramming of endogenous cardiac fibroblasts into cardiomyocytes and stimulation of endogenous cardiomyocyte



**Fig. 2** A combined approach for amelioration of injury and rejuvenation of cardiac tissue. Successful cardiac regeneration will likely necessitate a combination of therapeutic approaches. **(A)** Delivery of exogenous cells has been demonstrated *via* epicardial, intramyocardial (endocardial), intracoronary and intravenous routes. **(B)** Fibroblasts directly reprogrammed into cardiomyocytes either *in vitro* or *in vivo* can potentially serve as an abundant source of cells for cardiac regeneration. **(C)** Stimulation of native cardiomyocyte proliferation may be possible using a number of protein- and nucleic acid- based factors. Delivery of multiple cell types **(E)** along with delivery of biomaterials-based scaffolding **(D)** may be necessary for optimal cell engraftment and tissue regeneration.

expansion through growth and transcription factor delivery have the potential to sidestep the inherent limitations of exogenous cell delivery. Ultimate success with cardiac cell therapy will likely necessitate a combined strategy involving exogenous delivery of multiple complementary cell types, soluble factors for enhanced cell survival, concurrent stimulation of endogenous cardiomyocyte regeneration, recruitment and transdifferentiation of endogenous cardiac fibroblasts into cardiomyocytes through direct reprogramming, and the use of biomaterial scaffolds to provide structural support and biochemical cues during delivery (Figure 2).

## Acknowledgements

This work was supported by grants from the California Institute of Regenerative Medicine RC1-00354-1, the National Institutes of Health (NIH) DP2 HL127728 (R.A.) and NIH T32 HL78915 (C.H.C.).

## Conflicts of interest

The authors confirm that there are no conflicts of interest.

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