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Increases in plasma Tβ4 after intracardiac cell therapy in chronic ischemic heart failure is associated with symptomatic improvement

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Aim: Tβ4 is an integral factor in repair of myocardium in animal models. To investigate whether Tβ4 is important in human cardiac disease and has a role in mediating the beneficial cardiac effects of bone-marrow-derived stem cell (BMSC) therapy, we measured serial plasma Tβ4 levels in patients enrolled on the REGENERATE-IHD cell therapy trial. **Patients & Methods**: Plasma Tβ4 concentrations were measured in 13 patients who received BMSCs and 14 controls. **Results**: There was a significant increase in plasma Tβ4 in the BMSC group 24 h after intracardiac injection. Increases in Tβ4 levels were associated with improvement in New York Heart Association symptom class. **Conclusion**: This exploratory study highlights the need for further study of Tβ4 in human cardiovascular disease.

Keywords: ischemic heart failure • stem cell therapy • thymosin β4

The therapeutic use of bone-marrow-derived stem cells (BMSC) has been shown to be not only safe and feasible, but also associated with functional cardiac repair and improvement in left ventricular ejection fraction and symptoms in Phase I/II trials of acute and chronic myocardial ischemia. The largest meta-analysis to date demonstrates that cell therapy confers an approximate 4% improvement in cardiac function and infarct size [1]. The interim analysis of the REGENERATE-IHD trial [2] has reported safety and feasibility of combined administration of granulocyte-colony stimulating factor (G-CSF) followed by intramyocardial or intracoronary injection of BMSCs and a signal toward improvement in symptoms in patients treated with intramyocardial injection of cells compared with injection of serum alone [3].

A major criticism of clinical trials using cell therapy is that there remains a lack of understanding as to the mechanistic pathways that mediate cell-based therapy. It is now felt that transplanted cells take their effect in a paracrine manner by secreting soluble factors [4,5]. Adult BMSCs particularly mesenchymal stem cells (MSC) have been shown to produce and secrete a broad variety of cytokines, chemokines and growth factors that may be involved in cardiac repair [6], an effect that is upregulated during hypoxic stress [7]. Immediately following BMSC transplantation, tissue concentrations of these factors are seen to increase [8,9].

Tβ4 is a 43 amino acid polypeptide that has recently been implicated as a key player in cardiac repair and its known effects include wound healing, anti-inflammatory effects and angiogenesis [10–12]. Tβ4 is integral to embryonic myocardial development and in cardiomyocyte survival and conservation of ventricular function in adult murine models [13]. Landmark animal studies have revealed significant improvements in ejection fraction, cardiac volumes and scar size in animals pretreated with Tβ4 in experimentally induced myocardial infarction compared with sham-treated animals [14]. It is shown that Tβ4 can activate adult epicardium-derived progenitor cells usually quiescent in adult life, returning these cells to a less differentiated form and thereby restoring pluripotency [15]. It is entirely unknown as to whether Tβ4 is clinically significant in human cardiac disease.

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Tβ4 is shown to be expressed in subpopulations of BMSCs [16]. High levels of Tβ4 expression have been demonstrated in MSCs that have genetic modification with the prosurvival gene *Akt1* (Akt-MSCs), particularly under hypoxic conditions [4]. It is therefore possible to hypothesize that Tβ4 may have a pivotal role in mediating the beneficial effects of cell therapy.

We sought to determine whether plasma Tβ4 levels were affected by administration of cell therapy and whether this had an association with clinical outcome in a cohort of patients enrolled in the REGENERATE-IHD trial.

Patients & methods

Patient population

Consecutive patients recruited to the REGENERATE-IHD trial intramyocardial arm and intracoronary arm were also recruited to the Tβ4 substudy. The protocol was approved by the local Research Ethics Committee (REC no. 04/Q0603/13).

The study design, inclusion and exclusion criteria and primary and secondary end points of the ongoing REGENERATE-IHD trial have previously been described [3,17]. Briefly, patients with New York Heart Association (NYHA) class II–IV heart failure secondary to ischemic heart disease who are stable on optimal treatment are included in the trial. Patients are randomly allocated to intramyocardial arm, intracoronary arm or peripheral arm of the trial and further randomized 1:1 to either treatment or control group within each arm. All patients are given subcutaneous injections of G-CSF (10 μg/kg/day) for 5 days to mobilize BMSCs from the bone marrow. In the intramyocardial and intracoronary arms, this is followed by bone marrow aspiration, preparation of autologous BMSC and their intracardiac injection. In the intramyocardial arm, patients receive either intramyocardial injection of BMSCs or control (intramyocardial serum alone), while in the intracoronary arm, patients receive either intracoronary injection of BMSCs or control (intracoronary serum alone). The primary end point is change in left ventricluar ejection fraction at 1 year measured by cardiac computed tomography/MRI. Secondary end points that were reported on in the interim analysis [3] include change from baseline in NT-proBNP, a biochemical marker of heart failure, at 6 months and change in NYHA class from baseline at 6 months.

Blood sampling

One milliliter of blood was drawn at day 0 (D0, baseline), day 6 (after 5 days G-CSF pretreatment, prior to intramyocardial or intracoronary injections of BMSCs or serum alone) and day 7 (1 day post intramyocardial/intracoronary injection). Blood was drawn into EDTA-containing collection tubes and centrifuged at 1600g for 20 min. The plasma layer was aspirated and immediately stored at -80°C until Tβ4 measurement.

ELISA measurement of Tβ4 levels

The Tβ4 plasma concentration was measured with an ELISA (Immunodiagnostik AG, Bensheim, Germany). A dose–response curve of absorbance unit (optical density, at 450 nm) versus concentration was generated using the values obtained from standards and used to determine plasma Tβ4 concentrations. Due to significant interplate variation, plasma Tβ4 levels on D6 and D7 are expressed as a proportion of plasma Tβ4 levels on D0/at baseline. This was possible because all plasma samples from the same patient were performed on the same plate.

Statistical analysis

Continuous variables are shown as either median and quartiles or mean ±SD and categorical values are defined as percentages. The Student's t-test is used for the analysis of continuous variables and Pearson correlation coefficients are used to compare continuous variables and Tβ4 levels. A p-value of <0.05 was

considered to indicate statistical significance. Statistical analysis was performed using SPSS version 20.0 (IBM SPSS, Inc., NY, USA) and graphs produced using GraphPad Prism version 5.0 (GraphPad Software, CA, USA).

Results

From a total of 60 patients enrolled into the intramyocardial and intracoronary arms of the trial, plasma Tβ4 levels were measured in 16 patients in the intramyocardial arm and 11 patients in the intracoronary arm. Of the patients in whom Tβ4 level was measured, 13 patients received either intracoronary or intramyocardial injection of BMSCs and 14 patients received either intracoronary or intramyocardial injection of serum alone. Table 1 shows the baseline characteristics of the study population. There is no significant difference in past medical history, baseline cardiac therapies or baseline cardiac function and symptoms between the two groups.

Tβ4 levels after administration of intracardiac BMSCs or serum

The median plasma Tβ4 concentration at baseline (D0) for the whole group was 570.0 ng/ml (329.6–1148 ng/ml). Tβ4 concentration was measured on D6 and D7 as a proportion of each patient's baseline (D0) Tβ4 concentration. As shown in Figure 1, in the patients who received serum alone, there was no significant difference between Tβ4 level on D6 (prior to intracardiac injection) and that on D7 (after intracardiac injection). For the group of patients who received BMSCs, Tβ4 levels were significantly higher on D7 (after intracardiac injection) compared with D6 ($p = 0.0461^*$). There was no significant change in Tβ4 levels between D0 and D6 in either patients who received cells or those who received serum. Of the patients who received cells, there are four patients with a difference in fold change from D6 to D7, in other words, after cell reinfusion of ≥1. There is only one such patient in the serum group (Supplementary Table 1; see

Table 1. Baseline clinical characteristics of bone-marrow-derived stem cell and serum groups.

	Intramyocardial		Intracoronary	
	BMSC group (n = 8)	Serum group (n = 8)	BMSC group (n = 5)	Serum group (n = 6)
Age, years	67.1 ±10.5	59.3 ±12.2	62.0 ±8.9	63.8 ±6.6
Male, n (%)	8 (100)	8 (100)	5 (100)	6 (100)
Medical history, n (%)				
• Diabetes	3 (37.5)	2 (25.0)	1 (20.0)	2 (33.3)
• Previous MI	7 (87.5)	8 (100)	5 (100)	5 (83.3)
• CABG	2 (25.0)	2 (25.0)	4 (80.0)	1 (16.7)
• Time from last MI, years	9.3 ±7.6	9.6±9.7	11.8±7.4	8.4±6.6
• LVEF (%)	32.0 ±9.1	32.4±8.3	30.1±3.4	27.6±10.9
• NT-proBNP (pg/ml)	802 ±819	499±356	362±251	911±634
NYHA class, n (%)				
• NYHA 2	3 (37.5)	6 (75.0)	3 (60.0)	2 (33.3)
• NHYA 3	5 (62.5)	2 (25.0)	2 (40.0)	4 (66.7)
Medications at recruitment, n (%)				
• Statins	8 (100)	8 (100)	4 (80.0)	5 (83.3)
• ACEi/ARB	8 (100)	8 (100)	4 (80.0)	5 (83.3)
• β-blocker	8 (100)	7 (87.5)	4 (80.0)	5 (83.3)
• Aldosterone antagonists	7 (87.5)	4 (50.0)	4 (80.0)	3 (50.0)
• Diuretics	5 (62.5)	4 (50.0)	4 (80.0)	5 (83.3)
Devices, n (%)				
• ICD	7 (87.5)	4 (50.0)	3 (60.0)	4 (66.7)
• CRT	5 (62.5)†	0 (0.0)†	1 (20.0)	1 (16.7)

BMSC group vs serum group comparisons are not statistically significant unless otherwise noted.
†For CRT (n) p = 0.0256.
ACEi: Angiotension converting enzyme inhibitor, ARB: Angiotension receptor blocker; BMSC: Bone-marrow-derived stem cell; CABG: Coronary artery bypass grafting; CRT: Cardiac resynchronization therapy; ICD: Implantable cardioverter defibrillator; LVEF: Left ventricular ejection fraction; MI: Myocardial infarction; NYHA: New York Heart Association.

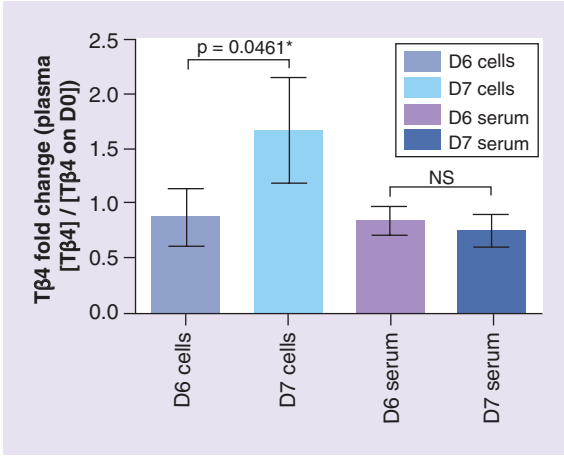

Figure 1. Plasma Tβ4 concentration on D6 and D7 expressed as a proportion of Tβ4 concentration at baseline (D0) for patients receiving either intracardiac bone-marrow-derived stem cells or intracardiac serum on D6.

D6: Day 6 – just prior to intracardiac injection; D7: Day 7 – 24h after intracardiac injection; NS: Not significant; Tβ4: Thymosin β4.

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Changes in Tβ4 levels, total cell count & cell delivery

The mean G-CSF-induced CD34+ cell count on D6 in the group that received BMSCs with a subsequent increase in Tβ4 level of ≥1 was 31.38 ±16.61/μl compared with 60.16 ±45.30/μl in the group where there was no change (p = not significant [NS]). The total number of CD34+ cells reinfused in the group with an increase in Tβ4 level of ≥1 was $3.275 \times 10^6 \pm 1.272 \times 10^6$ compared with $3.131 \times 10^6 \pm 1.762 \times 10^6$ in the group where there was no change (p = NS). In line with this, there were no significant differences between total mononuclear cell counts on D6 or total number of mononuclear cells reinfused on D7 between the two groups. Furthermore, there was no relationship between mode of delivery and fold change in Tβ4 levels between D6 and D7 (Supplementary Table 1).

Changes in Tβ4 levels & baseline cardiac function

Of the patients who received BMSCs and showed a fold increase ≥1 in Tβ4 between D6 and D7, the mean baseline left ventricular ejection fraction was 29.60 ±3.900%, compared with 31 ±8.451% in the BMSC group, where there was no increase in Tβ4 (p = NS). Similarly, there was no significant difference in mean baseline NT-proBNP between the BMSC group in which Tβ4 increased ≥1 and the BMSC group where there was no change in Tβ4 (450.5 ±214.4 pg/ml compared with 673.9 ±830.7 pg/ml respectively, p = NS). Baseline NYHA class did not differ significantly between the patients who had increases in Tβ4 and those who did not (Supplementary Table 1).

Changes in Tβ4 levels & clinical response to cell reinfusion

There was no significant correlation between increase in Tβ4 within 24 h of cell reinfusion and change in NT-proBNP at 6 months (Figure 2) as measures of clinical response. Five out of 13 patients who received cells experienced an improvement of NYHA class of ≥1 with the remaining not gaining any symptomatic benefit. The group that demonstrated an improvement in NYHA symptom class (responders) showed a significantly greater increase in TB4 levels after cell reinfusion than the nonresponder group, p = 0.0126* (Figure 3). In fact, all four patients with a difference in fold change from D6 to D7 ≥1 experienced improvement in NYHA symptom class at 6 months.

Discussion

Tβ4 is a major actin-sequestering peptide and has both intracellular properties being an important structural element of the cytoskeleton and extracellular properties that promote tissue repair and regeneration [18,19]. It has a ubiquitous distribution being expressed in all cell types except red cells and has been shown to have a role in angiogenesis with endothelial differentiation, tissue regeneration and reduction of inflammation in models of cardiac injury [13,20].

Tβ4 is felt to have the potential to improve cardiac outcome in the clinical setting of ischemic heart failure by promoting angiogenesis and tissue regeneration as well as preventing apoptotic death of cardiomyocytes and scarring [15]. Despite this, very little is known about the importance of Tβ4 in cardiac disease states or whether the marked improvements in left ventricular function that are seen in animals treated with Tβ4 has any clinical application.

Here for the first time we show that patients with chronic ischemic heart failure have detectable plasma Tβ4 levels (median 570.0 ng/ml, n = 27) and these are comparable with serum Tβ4 levels measured in patients with coronary artery disease, healthy controls and patients with liver failure from previously reported studies using the same methods. Healthy controls reportedly have significantly higher Tβ4 levels than all other groups (median 6504.7 ng/ml, n = 30) [21]. Patients with coronary artery disease both with and without good collateral supply also have higher Tβ4 levels (median 1024.5 ng/ml, n = 103; 1373.0 ng/ml, n = 87, respectively) [22]. Patients with acute on chronic liver failure and chronic liver failure, however, appear

to have similar levels of circulating Tβ4 to those of the cohort of chronic heart failure presented here (median 463.2 ng/ml, n = 30; 698.1 ng/ml, n = 31, respectively) [21]. Furthermore, higher Tβ4 levels are shown to correlate with survival [21]; similarly, levels of Tβ4 in bronchoalveolar lavage from patients with interstitial lung disease are positively correlated with improvement in lung damage [23]. Taken together, this suggests that there is a correlation between circulating levels of Tβ4 and extent of ischemia and resulting impairment of cardiac function; however, the mechanism underlying this is unknown.

In this study, we have measured circulating Tβ4 levels in chronic ischemic heart failure patients recruited to the REGENERATE-IHD trial comparing BMSC therapy to control. We have shown that in the cell-treated group, circulating Tβ4 levels were significantly increased within 24 h of receiving intracardiac infusion of BMSCs compared with the placebo group in whom circulating Tβ4 levels remained the same post intracardiac serum injection. There was no significant change in Tβ4 level over the time period during which G-CSF was administered (D0–D6). Tβ4 has been shown to be expressed in the MSC fraction of BMSCs [4] and therefore could be considered a candidate paracrine factor in mediating the favorable effects of BMSC therapy seen in acute and chronic ischemic heart failure. Here we show a marked fold increase in plasma Tβ4 levels in a small proportion of cell-treated patients, however, from these data, it is not possible to say what the source is. Our data show that there is no association between Tβ4 increase and circulating mononuclear cells or CD34+ cells secondary to G-CSF treatment. Neither is there a correlation with the total numbers of mononuclear cells or CD34+ cells injected. There is also no relationship between fold increase in circulating Tβ4 levels and mode of cell delivery, whether intracoronary or intramyocardial injection. Furthermore, analysis of baseline patient characteristics does not help to predict those patients in whom Tβ4 levels will increase. It may be that it is the interaction between the injected BMSCs and the myocardium that leads to the optimal hypoxic environment for the expression and production of Tβ4. However, it is also possible that Tβ4 is not produced in the myocardium as transplanted cells are known not to engraft within the myocardium. Increased circulating levels of Tβ4 may also be an effect of cells accumulating in other tissue such as thymus or spleen thereby increasing Tβ4 production.

While increased levels of circulating Tβ4 did not correlate with improvements in NT-proBNP levels, there was a significant correlation with improvement in symptoms. Those patients who showed increased circulating levels of Tβ4 within 24 h of intracardiac infusion of cells, all experienced improvements in NHYA symptom class at 6 months. In other words, the patients who responded to therapy had significantly higher Tβ4 levels immediately after BMSC infusion than those who did

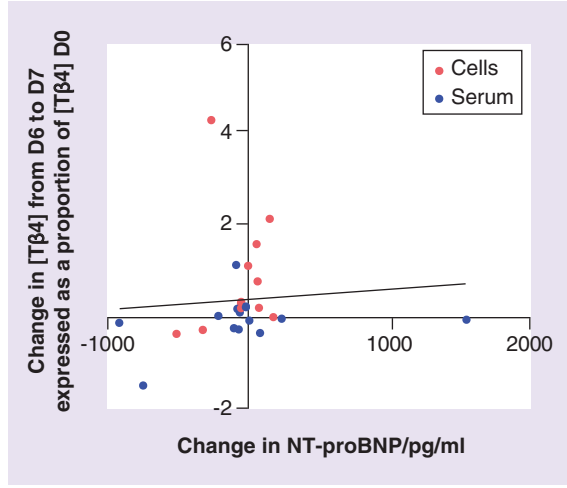

Figure 2. Correlation between fold change in Tβ4 between D6 and D7 and change in NT-proBNP at 6 months.

D0: Day 0 – baseline prior to 5 days G-CSF administration; D6: Day 6 – just prior to intracardiac injection; D7: Day 7 – 24 h after intracardiac injection; Tβ4: Thymosin β4.

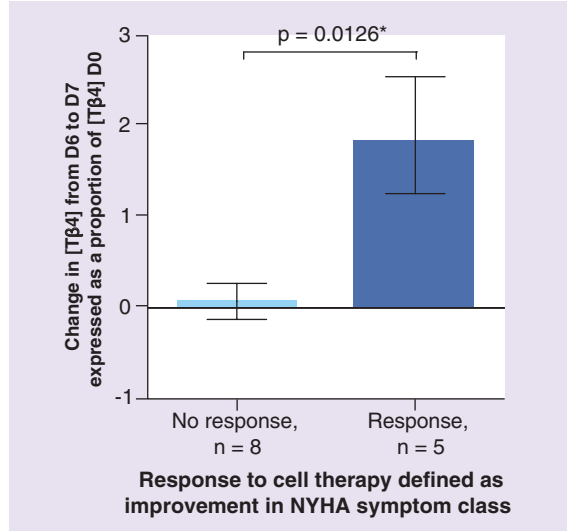

Figure 3. Change in Tβ4 levels in those patients who symptomatically responded to bone-marrow-derived stem cells according to New York Heart Association class at 6 months compared with those who did not symptomatically respond to bone-marrow-derived stem cells.

D0: Day 0 – baseline prior to 5days G-CSF administration; D6: Day 6 – just prior to intracardiac injection; D7: Day 7 – 24h after intracardiac injection; NYHA: New York Heart Association; Tβ4: Thymosin β4.

not. Whether this is merely a correlation without causative effect or in fact there is a mechanistic link between the two variables is unknown. It could be hypothesized that Tβ4 is the effector paracrine factor of BMSCs and patients who upregulate Tβ4 levels have an improved prognosis due to improved cardiomyocyte survival and more importantly Tβ4 could act locally on the epicardium to mediate neovascularization and myocardial regeneration. Recent reports showing increased circulating Tβ4 levels in patient with coronary artery disease and good compared with poor collateral supply [22,24] support a role for Tβ4 in angiogenesis within the adult myocardium.

Our data are exploratory and should be considered hypothesis generating in its utility. Firstly, only small patient numbers are analyzed meaning that only four out of 13 patients experienced fold increases of Tβ4 between D6 and D7 of ≥1. Further, interassay variation made it impossible to analyze absolute plasma Tβ4 concentrations and lastly the data were analyzed in a retrospective manner. Despite this, we have shown, for the first time, detectable circulating levels of Tβ4 in a cohort of patients with chronic ischemic heart failure. We have measured Tβ4 in the context of a randomized placebo-controlled autologous BMSC therapy trial demonstrating a cohort of patients to have both increased circulating Tβ4 levels in response to intracardiac cell infusion and improvement of NYHA symptom class.

Conclusion

This is a hypothesis-generating study of small sample size assessing the change in circulating Tβ4 levels in ischemic heart failure patients after receiving intracardiac BMSC therapy. We show that a subgroup of these patients show a significant increase in Tβ4 within 24 h of cell reinfusion. This increase in circulating Tβ4 levels while not associated with objective improvements in NT-proBNP level is associated with improvement of NYHA symptoms at 6 months. It is not possible to predict which patients will demonstrate a Tβ4 response to cell therapy based on patient characteristics or numbers of reinfused cells. While these are extremely small numbers, this is the first time such measurements have been made in this unique patient group and suggests that Tβ4 may have a role mediating the beneficial effects of cell therapy in cardiovascular disease.

Future perspective

Reports have shown Tβ4 to act as a modulator of cardiac repair in animal models. The molecular mechanism by which Tβ4 exhibits its cardioprotective effects is still not fully established. In order to enable translation of exciting animal and *in vitro* work into the clinical setting of cardiac regenerative medicine, it is essential to gain further understanding into the role of Tβ4 in human cardiac disease as well as how Tβ4 relates to existing cell therapies and novel cell types that are under investigation.

Executive summary

Background

- Tβ4: Thymosin β4 (Tβ4) is reported to be an important mediator of cardiac repair in animal models.
- We sought to assess circulating levels of Tβ4 in chronic ischemic heart failure patients recruited to the REGENERATE-IHD trial before and after intracardiac delivery of autologous bone marrow cells and correlate this with clinical outcome.

Results

- End-stage ischemic heart failure patients have detectable circulating Tβ4 levels.
- There is a significant increase in Tβ4 plasma concentration 24 h after intracardiac bone-marrow-derived stem cell (BMSC) administration compared with intracardiac serum administration alone (placebo).
- Increases in circulating Tβ4 levels are associated with improvement of New York Heart Association class symptoms at 6 months, although not with cardiac functional improvement as measured by *N*-terminal prohormone brain natriuretic peptide levels.
- Granulocyte-colony stimulating factor therapy does not produce a change in circulating Tβ4 levels in this patient group.
- It is not possible to predict in which patients circulating Tβ4 levels will be upregulated on the basis of patient characteristics, cardiac function or numbers of cells reinfused.

Discussion

- Circulating levels of Tβ4 compare well with previously studied cohorts with coronary artery disease and liver failure and are increased post intracardiac injection with BMSCs. These observations need validation in larger patient groups.
- The mechanism by which Tβ4 increases post BMSC injection and its relationship with improvement in symptomatic outcome require further investigation.

Conclusion

- Hypothesis-generating study supporting a role for Tβ4 as a mediator of BMSC therapy.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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Review

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Regenerative Medicine

Myocardial infarction: stem cell transplantation for cardiac regeneration

It is estimated that by 2030, almost 23.6 million people will perish from cardiovascular disease, according to the WHO. The review discusses advances in stem cell therapy for myocardial infarction, including cell sources, methods of differentiation, expansion selection and their route of delivery. Skeletal muscle cells, hematopoietic cells and mesenchymal stem cells and embryonic stem cells-derived cardiomyocytes have advanced to the clinical stage, while induced pluripotent cells are yet to be considered clinically. Delivery of cells to the sites of injury and their subsequent retention is a major issue. The development of supportive scaffold matrices to facilitate stem cell retention and differentiation are analyzed. The review outlines clinical translation of conjugate stem cell-based cellular therapeutics post-myocardial infarction.

Keywords: cardiac regeneration • bench to clinic • myocardial infarction

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A total of 11.2% of deaths worldwide are caused by ischemic heart disease according to the WHO statistics 2012 [1]. Ischemic injury of the heart causes loss of blood flow along the coronary arteries supplying the heart mainly affecting the flow to the ventricular portion of the heart. The relatively low regenerative potential of the resident cardiac stem cells (CSCs) is insufficient to replace the approximately 50 g of heart muscle, in other words, two billion cells that follows scar formation [2,3]. Infiltration of fibroblasts with the deposition of collagen and fibrin results in scar tissue formation [4]. The resultant damage leads to an increase in tensile strength, elongation and wall thinning of the heart, commonly known as 'infarct expansion' [5]. Details of the mechanisms are beyond the scope of this article and can be found in a thorough review by Pfeffer and Braunwald [4].

Pluripotent stem cells, are proliferative cells that can differentiate into cardiomyocytes fibroblasts, endothelial cells and smooth muscle cells are most sought after to replace post infarct scar tissue. There is also a need to arrest the progression of the infarct with various means immediately after the infarct. In this regard, adult bone marrow stem cells (BMCs) and mesenchymal stem cells (MSCs) have moved into extensive clinical trials, although CSCs and pluripotent stem cell-derived cardiovascular cell types are also showing promise. Methods such as cardiac restraints, hydrogels and patches have been proposed for the infarct condition and, while some of the methods are still in nascent stages of validation, others have reached clinical trials. Furthermore, scaffold materials are used to deliver cells temporarily or permanently to support the infarcted section of the heart. In this regard, scaffold materials are being envisaged as combination therapies along with stem cells and growth factors. The strategy required to mitigate the extent of damage due to infarction involves control and treatment at various levels of infarct progression. This can involve administration of anti-apoptotic agents in order to reduce cellular necrosis and resultant apoptosis that occur due to lack of oxygen [6]. The second goal should be the replacement of the scar tissue with cellular/molecular mediators that promote cardiac tissue regeneration. The

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primary goal should be to target the infarct as early in its progression as possible, ideally before scar tissue is formed. Clinical therapies look at the infusion of cells immediately after, in other words, 2–3 days to a week after the infarct and up to a year and have found that some cell types are more suitable than others. This review focuses on the various aspects of the sources of stem cells, their expansion and finally their clinical application in the stages of deterioration after a myocardial infarction (MI) episode. Various currently researched materials are compared and the criteria of the scaffold materials for cardiac applications are discussed.

Cell sources

There are different choices available to clinicians as given in Figure 1 for transplantation to the heart. Depending on the cell type in question the protocol will involve various steps of isolation, expansion and finally delivery as given in Figure 2. Endothelial cells and smooth muscle cells, the two most predominant cell types, must be represented in the cellular populations to be delivered. The modes of delivery of cellular payload can be systemic or localized for which various strategies of transplantation of cells have been elucidated. Resistance to ischemia is one of the major hurdles for stem cell populations to differentiate toward a cardiomyocyte population at the infarct site, especially with regard to electromechanical integration, and cellular retention.

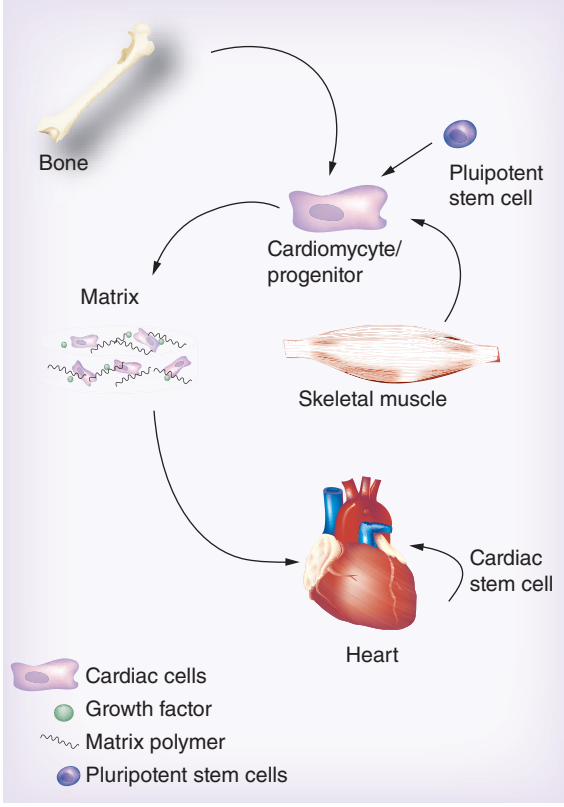

Figure 1. Cell sources and their methods of application for cardiac regeneration.

Adult stem cells

Skeletal muscle myoblast

Skeletal myoblasts (satellite cells) have been classically identified as a stem cell population resident within non-cardiac musculature. These can differentiate into various lineages, such as bone, cartilage and fat and are identified by the marker Pax7 [7]. Recently, non-satellite $CD34^-$, $CD45^-$ and $Sca1^-$ stem cell populations isolated from skeletal muscle cells have demonstrated rhythmic beating similar to cardiomyocytes in *in vitro* culture [8]. The autologous nature of these cells ensures their suitability for transplantation. These cells can further be modified to express markers like VEGF before transplantation into the heart.

Non-satellite skeletal myoblast cells, when transplanted into adult mice, have shown transdifferentiation into cardiac tissue [8]. The resistance of satellite cells to ischemia *in vivo* has resulted in better retention times as well as higher survival rates [9].

The suitability of autologous *ex vivo* expanded skeletal myoblasts to form viable muscle in severely scarred myocardium has been established [10]. It was also found that the catheter-mediated delivery of cells resulted in increased wall thickening at the target site and improved ejection fractions [11,12]. A consequent study reported a 3–8% change in the ejection fractions, even to the extent of ventricular remodeling [13]. Ongoing clinical trials are further looking into catheter-mediated delivery of cells [14].

The autologous nature of the satellite cells, along with the structural benefits that these cells endow, does create a case for the suitability of this stem cell population for transplantation. Nevertheless, there is doubt as to whether the cells provide only structural benefits rather than form new cardiac tissue, due to lack of trans-differentiation to cardiac tissue [15]. Furthermore, there are issues with engraftment; studies have reported low engraftment with over 90% injected cells dying within the first few days. A high number of cells, in other words, 600–800 million cells, when transplanted, have caused arrhythmia [16].

Adult bone marrow- & blood-derived stem cells

BMCs have been known to supply the entire repertoire of cells in the hematopoietic lineages, cardiomyocytes and various other lineages. Among the populations present, $Lin^-c\text{-}kit^+$, $CD133^+$, $CD133^-CD34^+$, $c\text{-}kit^+$ and $Sca1^+$ cells are found to be suitable for cardiac regeneration [17,18]. *In vitro* encapsulation of cells, within

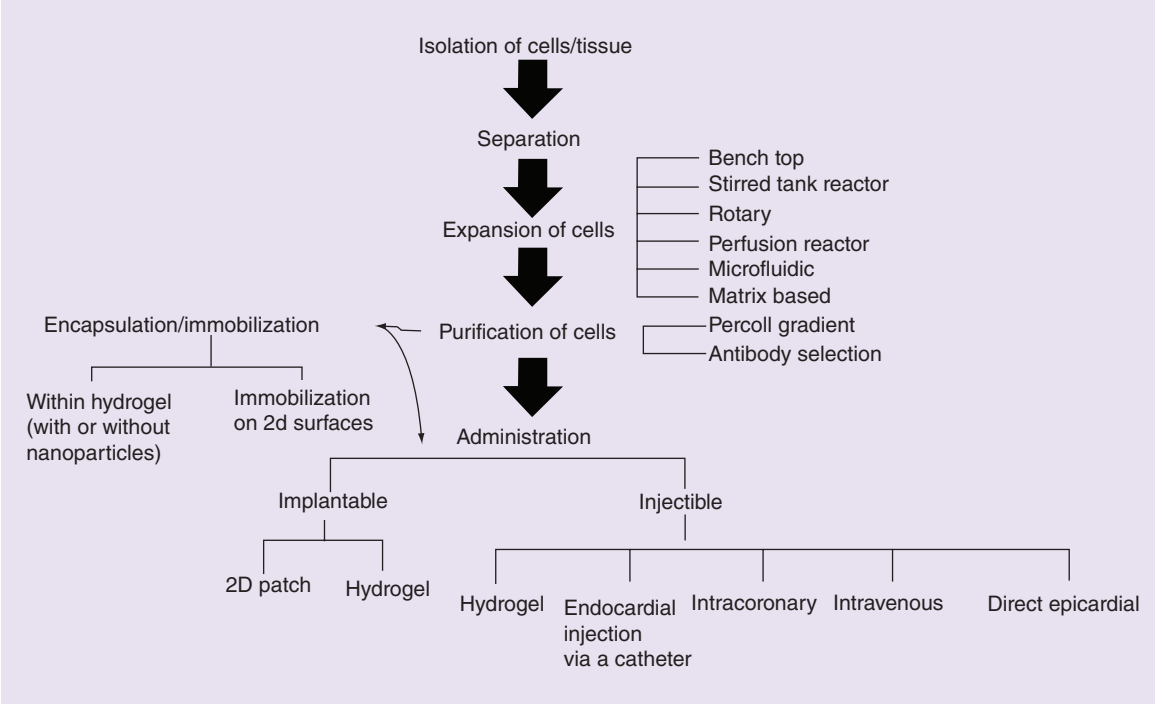

Figure 2. Strategies for cell therapy of cardiac tissue after a myocardial infarction.

porous type I collagen 3D conduits scaffolds resulted in expression of cardiac structural genes like α-myosin heavy chain (MHC) and β-MHC to sustained high levels for 28 days in culture [19].

c-kit$^+$ Sca1$^-$ cells improved survival, enhanced cardiac function, reduced regional strain, attenuated remodeling and decreased infarct size in mice [20]. While Lin$^-$c-kit$^+$ cells resulted in significant occupation of the infarct areas, when transplanted [18]. Other authors have suggested that the c-kit$^+$ BMCs do not fuse but differentiate to the endothelial and cardiac lineage after transplantation [21]. Canine models have been studied for a comparison of catheter-based endocardial to direct epicardial injections of blood-derived endothelial progenitors, as an alternative to intravenous administration of cells as shown in Table 1 [22]. Endocardial and epicardial administration routes of administration presented similar kinetics.

The TOPCARE-AMI clinical trial administered blood- and bone marrow-derived mononuclear cells post infarction with a beneficial effect through the prevention of remodeling [44,45]. Reversal of remodeling through paracrine signaling has been suggested as a probable mechanism [46]. Furthermore, bone marrow mononuclear stem cell administration has a negligible effect on left ventricular ejection fractions (LVEF), but a positive effect on remodeling at 6 months [47].

Improvements in ejection fractions varying from a minimum of 2% to a maximum of 7% have been reported with the administration of adult BMCs [48–51], but these improvements do not include left ventricular (LV) remodeling [52], local or global wall thickening [53] changes in LV end-diastolic volume and infarct size [54]. Other studies have demonstrated that these stem cells do not transdifferentiate into cardiomyocytes in an infracted heart [55]. Breitbach *et al.* have reported calcification and ossification at the infarct site, with the use of BMCs [56]. In studies using the CD34$^+$ cell population, retrieval of clinically relevant numbers is possible only through *in vitro* expansion before administration [57]. The CD133$^+$-purified hematopoietic stem cells (HSCs) when tested showed only limited improvement in cardiac function [17,58]. To promote further work in this area, ongoing clinical trials are trying to assess the efficacy further [59,60].

Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are defined by the expression of antigenic receptors for CD105$^+$/CD90$^+$/CD73$^+$, CD34$^-$/CD45$^-$/CD11b$^-$ or CD14$^-$/CD19$^-$ or CD79alpha$^-$/HLA-DR1$^-$-specific antibodies and their ability to differentiate into osteogenic, chondrogenic and adipogenic lineages [61]. Koninckx *et al.* have shown that TGF-β enhances the myocardial differentiation of bone marrow-derived MSCs by the expression of TnT in monoculture and MHC in coculture with rat neonatal cardiomyocytes [62]. They have also suggested that co-cultured hMSCs expressed the transcription factor GATA-4, but did not express Nkx2.5 [63]. 5-azacytidine (5-aza) or dimethylsulfox-

Table 1. Large animal models for myocardial infarction.

Animals	Cells	Route	Parameters for study	Outcomes	Ref.
Canine	Endothelial progenitor cells	Subendocardial and subepicardial injection	Comparison of subendocardial and subepicardial cellular retention and clearance kinetics	Similar subepicardial and a subendocardial technique kinetics	[22]
Canine	MSCs	Intramyocardial injection	MSC transplantation	Cells differentiate to endothelial cells and smooth muscle cells and enhance vascularization	[23]
Ovine	Autologous endothelial cell within fibrin matrix	Intramyocardial injection	Assessment of improvements through angiogenesis	Neovascularization improves blood flow, and improves left ventricular function	[24]
Ovine	Mouse cardiac committed ESCs	Intramyocardial injection	Cross species transplantation of committed ESCs	ESCs are immune privileged, and can improve heart function	[25]
Ovine	Allogenic STRO-3-positive mesenchymal precursor cell	Intramyocardial injection	Cell transplantation and dosage assessment	Attenuation of remodeling through vascularization	[26]
Ovine	Allogenic mesenchymal precursor cells	Intracoronary infusion	Safety, efficacy of transplant of cells	Infarct size decreased by 40%, blood vessel density increased by >50%, 8 weeks postinfarction	[27]
Porcine	BMSCs	Intramyocardial injection	Stem cell transplantation and cellular retention	Cellular retention better at infarct border zone	[28]
Porcine	Bone marrow-derived MSCs	Intramyocardial injection	Functional recovery after transplantation	Improve bioenergic and contractile function; improvements via paracrine effects	[29]
Porcine	Bone marrow-derived MSCs	Intramyocardial injections	Intracoronary catheter-mediated transplantation	Long-term engraftment, reduction in scar formation	[30]
Porcine	ADSCs and BMSCs	Intracoronary injection	Comparison of ADSCs and BMSCs	Improvement in cardiac function via angiogenesis	[31]
Porcine	Allogenic bone marrow MSCs	Intramyocardial injection	Assessment of improvement in left ventricular function	Improved vascularization through differentiation into cardiomyocytes and endothelial cells	[32]
Porcine	Bone marrow-derived MSCs	Transendocardial injections	Stimulation of CSCs to proliferate and differentiate	20-fold increase in endogenous c-kit$^+$ CSCs	[33]
Porcine	MSCs	Intravenous injection	Infusion of cells immediately after infarct hypothesized to reduce remodeling	Improves left ventricular ejection fractions, prevents wall thickening in noninfarcted myocardium	[34]
Porcine	Allogenic MSCs	Endomyocardial injection	Safety of endomyocardial delivery and dosage	Reduction of infarct size attributed to paracrine effects	[35]
Porcine	MSCs	Intracoronary infusion	Assessment of localization of MSC	Remodeling prevented up to 2 months after myocardial infarction	[36]

ADSC: Adipose tissue-derived stem cell; BMSC: Bone marrow-derived stem cell; CDC: Cardiosphere-derived stem cell; CS: Cardiosphere; CSC: Cardiac stem cell; ESC: embryonic stem cell; hCSC: Human cardiac stem cell; hMSC: Human mesenchymal stem cell; MSC: Mesenchymal stem cell.

Table 1. Large animal models for myocardial infarction (cont.).

Animals	Cells	Route	Parameters for study	Outcomes	Ref.
Porcine	Peptide-1-eluting MSCs	Intracoronary infusion	Study of engraftment, survival and dosage	Improvement in ejection fraction by 9.3%, alginate facilitates retention of cells	[37]
Porcine	Autologous MSCs	Intracoronary perfusion	To assess whether MSCs mobilize bone marrow progenitors to myocyte proliferation	Increase in circulating c-kit$^+$ and CD133$^+$ cells with increase in myocardial c-kit$^+$/CD133$^+$ and c-kit$^+$/CD133$^-$ bone marrow progenitor cells	[38]
Porcine	ADSCs	Intracoronary, transendocardial	Comparison of modes of delivery of cells	Both modes are similar in engraftment and differentiation, intracoronary mode induces neovascularization	[39]
Porcine	Allogenic ADSCs	Intracoronary	Assessment of immune response	Illicit an immune response	[40]
Porcine	Allogenic CDCs	Intracoronary infusion	Safety and efficacy of CDCs as adjunctive therapy to reperfusion	Infarct size reduction, attenuation of remodeling, microvascular occlusion prevention	[41]
Porcine	CS	Transendocardial injection	Safety, efficacy and dosage of CSs injection	Regeneration through increase in viable myocardium	[42]
Porcine	hCSCs/hMSCs, hCSCs alone, hMSCs alone	Intramyocardial injection	Combination of hMSCs with c-kit$^+$ CSCs hCSCs produces greater infarct size reduction	Twofold reduction infarct size in hCSCs/hMSCs as compared with, hCSCs alone and hMSCs alone	[43]

ADSC: Adipose tissue-derived stem cell; BMSC: Bone marrow-derived stem cell; CDC: Cardiosphere-derived stem cell; CS: Cardiosphere; CSC: Cardiac stem cell; ESC: embryonic stem cell; hCSC: Human cardiac stem cell; hMSC: Human mesenchymal stem cell; MSC: Mesenchymal stem cell.

ide induce rat MSC differentiation toward cardiomyocytes, but have no such effect on hMSCs [64,65]. BMP-2 or FGF-4 have also been used to enhance the differentiation potential of rat MSCs *in vitro* [66]. Novel ways in which entrapment of cells within hyaluronic acid-based 3D scaffolds have demonstrated that cell spreading occurs when there are matrix degradation moieties present within the scaffold, especially in the presence of the RGD peptides [67]. Furthermore, matrix stiffness has been shown to be a key factor in MSC proliferation within fibrin scaffolds [68].

Some studies suggest that MSCs, when injected intramyocardially, differentiated to vascular smooth muscle cells or endothelial cells *in vivo* and showed improvements via angiogenesis in a porcine model of ischemia [69]. Furthermore, human umbilical cord blood-derived MSCs, when transplanted into mice, resulted in improvements through paracrine effects [70]. Preconditioning of these stem cells with 5-aza resulted in differentiation of MSCs to cardiomyogenic cells, when transplanted into mouse models of MI; this prevented infarct expansion and eventually improved heart function [66,71]. BMP-2 and FGF-4 can alternatively be used for the differentiation of MSCs toward cardiomyocytes. Studies report that BMP-2- and FGF-4-treated MSCs, when transplanted into rat models, demonstrated improvements similar to 5-aza-treated cells [66]. Contrary to claims made about the lack of differentiation potential of MSCs, studies have shown that they could differentiate to cardiomyocytes or fibroblast scar tissue, when transplanted in rats [72]. Furthermore, adipose-derived MSCs (ATMSCs) have been used as cell sheets to repair the infracted myocardial cells in rats, resulting in the reversal of wall thinning of the myocardium [73]. ATMSCs have induced vascularization with VEGF expression, additionally eliciting an immune response [39,40]. Cellular retention studies in porcine animal models transplanted with bone marrow-derived MSCs have indicated that infarct border zone injection retained more cells than direct injection into the heart [29]. Cardiac functional improvements in porcine models after transplantation of bone marrow-derived MSCs have attributed improvements to paracrine effects, while reporting retention of as low as 0.035% cells at the infarct site after peri-infarct injection of cells [28]. A study by Toma *et al.* has shown that hMSCs, when injected intraventricularly into SCID mice, differentiated into cardiomyocytes with the expression of cardiac-specific TnT, α-MHC, α-actinin and phospholamban with visible-striated fibers [74]. Furthermore, ablation of proinflammatory receptors TNF-α on MSCs has been linked to increased survival and reduced infarct size [75].

Clinical studies have shown that bone marrow-derived MSCs are safe for use through the transendocardial route of administration [76]. Intravenous transplantation of allogenic hMSCs at various single dosages of 0.5, 1.6 and 5 million cells/kg resulted in marked improvements with reduction of arrhythmias and improved LV function but no dosage response for most parameters [77]. Clinical studies have shown that the administration of MSCs to the heart leads to a therapeutic result via a paracrine effect rather than differentiation of MSCs to cardiomyocytes, while others have suggested differentiation toward a lineage based on the environment. To realize the full potential of MSCs as therapeutic agents, their differentiation to cardiomyocytes, in order to replace the cellular losses caused due to an infarct, is vital. Cardiomyocytes as well as angiogenic progenitors, if produced by the MSCs, will replenish the cells from the depleted heart and increase circulation to the affected area. Clinical studies are underway to assess the comparison of transplantation of autologous hMSC transplantation versus allogenic hMSCs, transendocardially [78,79].

Cardiac stem cells

The presence of a self-renewing, clonogenic and multipotent population of cells within the heart that is induced by paracrine signaling in the event of ischemia, has been established [80]. However, these cells cannot overcome the local loss of cells after an infarct [81]. The renewal rate of these cells declines at the rate of 1% per year at age 25 years to 0.45% at age 75 years [82,83]. The different cell populations isolated and characterized are c-kit$^+$ cells, Sca1$^+$ (CD31$^-$) cells, isl-1$^+$ (c-kit1$^-$ and Sca1$^-$) cells and cardiosphere-derived cells [84]. Cardiospheres (CSs) are clusters of self-adherent cells formed when heart biopsy specimens are expanded *in vitro* [85]. The core of the CS is composed of c-kit$^+$ cells, while cells that exhibit endothelial and stem cell markers (Sca-1, CD34 and CD31) are on the periphery [86].

Sca1$^+$, when induced by 5-aza-C [81] or oxytocin [87], result in the expression of cardiac transcription factors cardiac troponin1, sarcomeric α-actin, MHC and Nkx2.5. Oxytocin induces differentiation of the Sca1$^+$/c-kit$^+$ population to cardiomyocytes. Sca-1$^+$/CD31$^-$ cells differentiate to cardiac myocytes and endothelial cells in the presence of FGF, 5-aza-C and Wnt antagonist Dkk-1 [87]. Extracellular matrix (ECM) stiffness can induce differentiation as well; a matrix modulus of 31–35 kPa can support CSs and results in a high expression of cardiac markers cardiac TnT (cTnT) and cardiac MHC (MYH6) [88]. FGF-2 has been shown to play a critical role in the mobilization and differentiation of resident cardiac precursors in the treatment of cardiac diseases *in vivo* [89]. c-kit$^+$ cells have been found

to solely mitigate regeneration of a damaged heart [90]. They also induce neovascularization on transplantation via a paracrine effect [87,91–92]. Oxytocin-activated c-kit$^+$ cardiac progenitor cells, when injected at the site of coronary occlusion, differentiate to smooth muscle cells and endothelial cells [93]. Sca1$^+$ cells, on the other hand, show connexin 43, cTnI and sarcomeric α-actin expression after intravenous infusion into mouse hearts following ischemia/reperfusion [81]. Cardiosphere-derived cardiac progenitor cells contribute to improving ventricular function in mouse and swine models [86,94–95]. Furthermore, these cells do not induce immune reactivity, when transplanted [96]. Coronary infusion of CDCs in porcine models also provides a good model for the safety of the delivery of cells, modes of delivery as well as the benefits of such delivery [41].

Autologous c-kit$^+$ CSCs isolated from the right atrial appendage have been clinically administered in the SCIPIO trial through coronary infusion into patients after expansion [97]. LV ejection fraction increased from 30 to 38% and the infarct volume decreased from on average 32.6 to 7.2 g within 4 months of infusion [97]. However, doubts were cast as to why results were published before the trial was completed, further as to why patient from the non-randomized part of the trials were analyzed and results displayed [98]. Controlled double-blinded and randomized trials overturning positive results of non-randomized or partially randomized trials were cited to be the reason behind the objections. A separate study has harvested CDCs after generating CSs from end myocardial autografts and demonstrated reduction in scar mass and increase in viable tissue in the Phase I CADUCEUS clinical trials [99]. Follow-up studies with the patients revealed increase in viable myocardium, consistent with regeneration; furthermore, patients 1 year after MI are also eligible for the treatment and show improvements similar to those treated 2–3 months post-MI [100,101].

Clinical relevance of CDC transplantation is possible only after autologous cardiac tissue is harvested from patients during procedures like coronary artery bypass grafting (CABG). Although the CADUCEUS Phase I clinical trials points to improvements of LVEF over bone marrow cell transplantation and the SCIPIO trial of the improvements due to c-kit$^+$ cells, there is further need for clinical data to ascertain the efficacy of these cells [99]. Recently, van Berlo *et al.* cast doubts on the actual effective populations of c-kit$^+$ cells to mediate a regenerative response [102]. But the effectiveness of the cre-lox recombination system used to come to their conclusions has been elaborated [103]. Furthermore, the benefits of double-blinded, randomized and placebo-controlled clinical trials have to be understood to design effective clinical trials.

Pluripotent stem cells

Embryonic stem cells

Embryonic stem cells (ESCs) are cells isolated from the inner cell mass of blastocysts and which can give rise to the three germ layers, as well as giving rise to all the cardiac subtypes. ESCs have demonstrated differentiation toward a cardiac lineage and expression of cardiac functions [104–110] and further to prove their proliferative capacity, since a large number of cells are required at the site of infarct [111,112]. *In vitro* differentiation of ESCs has been optimized in mouse cell lines as well as human; while some protocols of differentiation work for mouse cell lines, some others work for human cell lines [113]. Furthermore, the use of gelatin, agarose and poly(lactide-*co*-glycolide) (PLGA)-based microparticles within cellular aggregates for differentiation has improve gene expression [114]. Apart from simple spontaneous differentiation protocols, to usage of mediators like BMP-4 and activin A and to coculture pluripotent stem cells with endothelial cell lines (END-2) have been used for the direct differentiation of ESCs to the cardiac lineage and to improve the yield of cardiomyocytes population generated therein [106,115–116]. ECM material stiffness is another aspect that is being studied to direct differentiation. A study showed that a dynamic module of ~8.6 Pa is suitable, and that differentiation was better in the presence of ECM as against collagen hydrogels supplemented with cardiac growth factors alone [117]. Hyaluronic acid/polyethylene glycol (PEG) hydrogel scaffolds with a dynamic modulus ranging from 1 to 8 kPa influenced differentiation of chicken embryonic cells [118]. Differentiation toward a cardiac lineage has led to the production of ECM proteins versican and hyaluronan [119]. While differentiated cells migrate toward fibronectin and noncanonical Wnt gradients [120]. Taken together methods of differentiation, isolation, enrichment and storage have been optimized to facilitate transplantation [121]. Allogenic transplantation of undifferentiated ES cells did not lead to a cardiomyocyte fate in either normal or infarcted hearts, neither was an allogenic immune protection observed. However, xeno transplants of cardiac-committed mouse ESCs into ovine models have proved that ESCs are immune privileged, as shown in Table 1 [25]. Cardiomyocytes derived from human ESCs have been able to repopulate rat hearts, suggesting an encouraging scenario for their use with humans [122]. Guinea pig injury models have shed light on the protective effects of transplanted ES-CMs against arrhythmias while beating in sync with host cardiac tissue [123]. Frozen human ESCs-derived cardiomyocytes could be revived and administered to nonhuman primates, leading to remuscularization and electromechanical integration albeit with the occurrence of nonfatal arrhythmias [124]. Matrix-

impregnated ES-CMs have utilized matrix properties to revascularize host tissue while controlling the immune response [125], Ongoing clinical trials are testing the use of fibrin gel embedding human ESC-derived CD15$^+$ Isl-1$^+$ progenitors [126].

Induced pluripotent stem cells

With the advent of induced pluripotent stem cells (iPSCs) in 2006 [127], a new opportunity presented itself toward the generation of pluripotent ES-like cells from somatic cells. It was shown that normal somatic cells could be converted to what are known as 'iPSCs' by the forced expression of four crucial factors transcription factors: Oct4, Sox2, c-Myc and Klf4 [127]. This technology has proved itself by its application across various species and tissues [128]. iPSCs too have shown properties of differentiation similar to ESCs [129–133]. Although differentiation protocols have succeeded in increasing the efficiency of differentiation, a cause for concern with respect to the final administration of iPSCs is the undesirable transfer of pathogens and ethical approval for transfer of cells cocultured with other cells lines [134], and third isolation of cardiomyocytes from the undifferentiated population [135]. Immunological safety of iPSCs were raised by Zhao *et al.* [136], but implanted tissue grafts obtained from iPSCs-derived cells implies that these cells are safe to take to the next level in tissue engineering of patient-specific cells [137]. Furthermore, cardiomyocytes, endothelial cells and smooth muscle cells derived from these cells have been tested on porcine infarct models, along with fibrin-encapsulated IGF. The results indicate a substantial reduction in infarct size, ventricular wall stress and apoptosis [138].

Various aspects of cardiac regeneration such as effective differentiation of stem cells, electrical and mechanical integration and especially long-term effects without adverse side effects – are yet to be dealt with in addressing the issue of regeneration of the heart at the site of MI [139]. Although it has been indicated that hESC-CMs and hiPSC-CMs 80–120 days in culture compare well with host cardiac tissue enough to elicit better integrative effects on transplantation [140]. Novel protocols of reprogramming fibroblast with cardiac genes *Gata4*, *Tbx5* and *Mef2c* have resulted in fibroblasts with limited survival and low cardiac molecular or electrophysiological change [141]. To further the iPSCs potential, the Japanese government recently gave permission for the conducting of clinical trials for the treatment of macular degeneration using iPSCs, suggesting a paradigm shift toward the use of iPSCs for therapy [142]. To harness ES cell potential, somatic cell nuclear transfer (SCNT) or somatic cell reprogramming offers a solution for the isolation of patient-specific cells for treatment. Furthermore, pluripotent stem cells generated from parthenogenesis have shown potential toward cardiac differentiation and efficient integration within host tissue [143].

Combinational therapy

Graft transplantation strategies required to address the reduction of vascular endothelial as well as smooth muscle cells to effectively address the site of infarct. Combined cell approaches like the transplantation of skeletal muscles and BMCs have indicated improvements in LVEF in the combined group against the skeletal muscle only group [144]. Human CSCs (hCSCs) c-kit$^+$ combined with bone marrow MSCs (hMSCs) administered to porcine MI models brought about a twofold greater reduction in the infarct size as compared with the use of the cell populations alone [43]. Further studies report that the administration of just bone marrow MSCs results in a 20-fold increase in c-kit$^+$ CSCs to synergistically mediate improvements [33]. Pluripotent stem cell differentiation to cardiac subtypes and transplantation of cardiomyocytes, endothelial cells and smooth muscle cells population together served to compensate losses to muscle as well as vasculature [138]. The transplantation of multiple cell types opens up an undiscovered area of cell therapy with the potential to study synergistic effects of complimentary cell population in MI therapy. A complementarity between the cells also gives the opportunity to reduce the final number of cells administered along with benefits greater than administration of each of the cell types alone [144].

Modes of application of stem cells in myocardial infarction

Various modes of delivery of cells to the site of infarct have been discussed extensively by Jezierska-Woźniak *et al.* and the resulting inefficiencies of the methods involved [139]. There have been issues with retention of cells as well as homing of cells, with methods like intravenous infusion [145], intracoronary injection [146] and direct epicardial [147] or endocardial injection via a catheter [148,149]. Although catheter-based clinical trials for transplantation of skeletal myoblast show improvements in the infarcted heart [11,150], there are other studies that suggest a completely contrary scenario to the transplantation of these cells [15]. A method that will allow a small population of progenitor cells either unipotent, multipotent or pluripotent to be encapsulated and delivered to the site of infarct is desirable. This will facilitate retention until differentiation, create a barrier between the undifferentiated population and the adult cells, preventing any adverse effects due to the undifferentiated population and

reduce the final cell number required for transplantation. This should facilitate paracrine effects, if any, without the harmful effects of the delivered cells, such as ossification and calcification. Furthermore, there is a need for direct contact of the tissue with the delivered material and cells.

Implantable systems

Cardiac patches

2D approaches have been pioneered in order to have strict control on the constructive elements that go into the scaffold, namely growth factors, cells and small molecules. Cardiac patches were developed to place elastic support with/without cells along the external ventricular wall of the myocardium for regeneration. ECM collagen has been used to prepare patches for treatment of MI by the transplantation of $CD133^+$ cells. Although there was visible angiogenesis at the site, the cells failed to differentiate to cardiomyocytes [151]. Polyurethane (PU) and poly(ester urethane) (PEU) rubbers are suitable candidates for the heart [152,153]. When cardiomyocytes were grown on biodegradable polyester urethane urea (PEUU), the membrane could contract the patch [154]. Other studies have shown that phytic acid cross-linked peptides, prepared by electrospinning, mimic the ECM in the heart [155]. Mouse iPSCs-derived cardiomyocyte cells have been used to prepare tissue sheets on thermoresponsive polymers [156]. Poly(glycerol sebacate) (PGS), another material whose mechanical characteristics can be tailored to match the heart, promoted the growth and beating of ES cell-derived cardiomyocytes *in vitro* [157]. Constructs with a combination of polytetrafluoroethylene, polylactide mesh, and type I and IV collagen hydrogel have been used to encapsulate MSCs [158].

PU is elastic and degradable *in vivo*. Animal trials of biodegradable PU-conducted patches promoted contractile phenotype smooth muscle tissue formation and improved cardiac remodeling and contractile function at the chronic stage [154]. iPSc-derived tissue sheets, when implanted in mice, reduced LV remodeling [156].

Poly(tetrafluoroethylene) reinforced porous poly(L-lactic acid) mesh seeded with bone marrow-derived mesenchymal cells and soaked in type I and IV collagen were sutured onto the rat infarct wall after a ventriculotomy. This resulted in a reduction in aneurysm elongation [158].

Ex situ gelled: hydrogel scaffolds

Hydrogels have been widely used as their mechanical properties can be fine-tuned to match those of cardiac tissue. Table 2 compares the stiffness of various gels and the cardiac matrix.

Hydrogels with stiffness lower than heart tissue can be used as temporary space-filling moieties, and further can be used to deliver stem cells and/or molecules for growth. In this regard, collagen injections into the ventricular wall have been shown to prevent progressive wall thinning, a sequel to permanent heart dysfunction, in rats [171]. Furthermore, hydrogels made up of ECM and collagen were able to differentiate human ESCs *in vitro* to cardiomyocytes [117]. Growth factor bFGF, along with MSC delivery, was demonstrated by encapsulating within thermoresponsive N-isopropylacrylamide (NIPAAm), N-acryloxysuccinimide, acrylic acid and hydroxyethyl methacrylate-poly(trimethylene carbonate). These hydrogels were able to sustain the growth of the cells through bFGF release [172]. bFGF has also been used for improvement in vasculature by Iwakura *et al.* [173].

ESCs encapsulated in collagen type I transplanted into intramural pouches at the infarct wall, resulted in reduction of fractional shortening. Carbohydrate polymers, like alginate, have been used for seeding cells and further implantation into mice to prove their efficacy as carriers for cells. These implants reduced LV remodeling, and it is further proposed as a carrier scaffold for iPSCs [174]. Zimmerman *et al.* have developed tissue by casting a mixture of collagen type I along with neonatal rat cardiomyocytes into moulds to form engineered heart tissue (EHT). These constructs were developed into ring-shaped flexible structures and sutured onto pericardiectomized rat hearts [175]. The EHT transplant became vascularized and electrically integrated *in vivo* and since these were prepared in serum-free media conditions, immunosupression was not required during transplantation [175,176]. Engineered heart muscle was developed with a similar approach by assembling cardiomyocytes derived from the differentiation ESCs onto EHT [177].

Of all the constructs developed, the ones that were successful were those derived from native heart tissue. Furthermore, collagen types I and IV have also been successful in being able to support cellular growth, cellular vascularization and to allow electrical integration within the heart. In case of transplantation of pluripotent stem cells, it will be essential to differentiate these on site with molecular mediators entrapped within the hydrogel; alternatively, one could use the stiffness characteristics of the hydrogel to differentiate the cells. Although robust, the hydrogel approaches can be employed only by surgical intervention.

Injectable systems

In situ gelling systems

Implantable systems can only be administered through invasive surgical intervention. Thus, implantation of these constructs will have to accompany procedures like CABG. *In situ* gelling systems, on the other hand,

Table 2. Matrix molecules and the relevant stiffness they can provide.

Congestive heart failure	Material stiffness	Ref.
Fibrin	50 Pa	[159]
Matrigel1	30–120 Pa	[160]
Type I collagen gels	20–80 Pa for 1–3 mg/ml	[161]
N-isopropyl acryl amide	100–400 Pa	[162,163]
Alginate	100 Pa to 6 kPa	[164]
Polyethylene glycol	1–3 kPa	[165]
Heart	50 kPa in normal hearts or 200–300 kPa in congestive heart failure hearts	[166–170]

are defined by a sol-to-gel transition from *in vitro* to *in vivo* setups, respectively. This method of gelation can assist the administration of the gelling polymer through a catheter, facilitating a minimally invasive method to cardiac treatment. In regard to this, the materials that have been studied extensively are fibrin glue [178,179], collagen [171], matrigel [180], hyaluronic acid [181], keratin [182], ECM [183,184], alginate [174,185]. There are many potentially useful materials that can fulfill this role and are yet to be tested in this application. Endothelial cells home to a self-assembling injectable RAD16-II peptide scaffold and cause more angiogenesis as compared with matrigel. Potential myocyte progenitors also populate the peptide microenvironment created *in vivo*, and the retention of myocytes is higher as compared with matrigel [186]. Furthermore, this study demonstrated that ESCs spontaneously differentiated to αMHC-positive cells *in vivo* within the peptide scaffold. Cell survival was better within fibrin glue when delivered through injectable fibrin glue scaffolds compared with the cellular cardiomyoplasty technique, additionally inducing neovascularization and reducing infarct expansion [179]. This was followed up with a study that suggested short-term improvements of the alginate fibrin blends at the site of infarct [187]. *In vivo* studies via injection through a catheter to a rat heart demonstrated the injectability of a porcine heart-derived matrix as well as endothelial cell infiltration within the matrix [183,184]. The method of delivery has been known to induce improvements within the cardiac environment with and without bone marrow mononuclear cells when injected with fibrin, collagen and matrigel, albeit separately [188,189]. Other methods have been studied, such as collagen through catheter encapsulated with bone marrow cells [190] and without cells [171]. Both these studies showed improvement in LV function without vascularization, but in the study by Huang *et al.*, there was also an improvement in vascular density [189]. Another widely available tissue culture matrix called Matrigel™, has been used as an *in situ* gel. Studies have demonstrated improvement in LV function with the gel, and ESC delivered along with it caused increased vascularization at the site of infarct [191,192]. Simulation of injection of material to the heart injected at various sites postinfarct suggests that administration of a noncontractile material at the site of infarct helps reduce stresses on the myocardium [193]. Self-assembling peptides have been useful in the delivery of IGF to the heart and permit the sustained release of the growth factor along with aiding the positive effects accrued to the cells delivered along with the peptide matrix [194]. Ungerleider and Christman have dealt with injectables and large animal models in detail, and according to their opinion, shorter gelling times are the not suitable for the delivery of injectable gels through catheters [195]. Furthermore, expansion and encapsulation through current good manufacturing practices, if not performed with adequate robustness, result in inefficient scaffolds. Despite positive results on a range of materials as injectables, alginate without cells is being currently clinically tested for its efficacy to prevent ventricular remodeling [196–198]. Radhakrishnan *et al.* have emphasized the importance of appropriate mechanical properties and electrical conductivity of the polymers used as injectables to be important in their overall regenerative potential [199].

Translational & future perspective

With the innovations in cardiac support devices to provide care immediately after an infarct and to prevent cardiac remodeling, it was envisaged that the devices and innovations market in the cardiac space would get a boost [200,201]. But after a 10-year battle with the US FDA, the cardiac mesh support device has not seen the light of day, even after positive clinical results. Regulations are established for implantable devices, like cardiac stents, valves, pace makers and LV assist devices (LAVD) such as HeartMate® I and II, CentriMag, SynCardia Total Artificial Heart. However, there is no regulation for implant materials, with or without cells, to mitigate therapy. On the

other hand, heart injectable regulations are structured toward delivery of small molecules via intracoronary, intracardiac injections or with transcatheters [195].

Most present clinical trials are performed with established autologous stem cell populations. Although these have accrued benefits, the loss of cells, paracrine effects and differentiation away from the cardiac lineage are an associated issue with transplantation ESCs have proved their immune privilege, their propensity for teratoma inhibits their usage, but a differentiated population of committed ESC-derived cardiomyocytes are a useful proposition as a cell source. Although a significant hurdle therein is the efficient differentiation toward a cardiac lineage, obtaining functional and viable cells after differentiation and most importantly electromechanical integration with host tissue after implantation is desired. iPSC technologies, on the other hand, are yet to be realized in their complete potential and require more work for application. Real-time monitoring of cells within embedded matrices has been made possible, through nanoparticulate approaches giving us robust tools to monitor and assess tissue regeneration *in vivo* [202]. This will surely bring down investment of time into the selection of cells.

Bioreactor systems are being optimized with suitable conditions for the expansion and differentiation of stem cells [203]. Devices, such as those prepared by Kofidis *et al.* [204] and Ting *et al.* [205], can be used for simultaneous expansion and differentiation of cells *in vitro* to prepare grafts for transplantation *in vivo*. Antibody purification of cardiomyocytes is available through antibody to SIRPA, resulting in a high efficiency for selecting cardiomyocytes [206]. These cells can further be encapsulated to prepare a 3D architecture and then delivered, or grown, in a 2D matrix and layered to have a 3D structure for implantation. Good manufacturing practice requires that the growth, propagation and differentiation of cells for commercial use have to be done with animal product-free material [207].

Implant material properties of toxicity, biodegradability and physical characteristics, like stiffness, are established in the literature. Implantation of patches, cardiac assist devices and injectable noncontractile supports have been studied. Cardiac support patches can be administered in the event of superficial scarring of the heart, leading to loss of contractile tissue. Injectable hydrogels accompanied with cells can also be administered at the border zone to prevent remodeling due to scarring. Furthermore, reperfusion procedures, such as CABG, can be accompanied with such implantation of hydrogel grafts at multiple sites along the epicardium. This, along with reperfusion, will facilitate the ingrowth of stem cells and their final differentiation to cardiomyocytes. Additionally, with degradable materials, it is possible after a period, the cells will be the only remnant of the procedure. Injectable materials like self-assembling peptide matrices, for example, RAD-16, fibrin glue, alginate, agarose can be administered via a transcatheter system, or normal cardiomyoplasty, epicardially or endocardially. Pluripotent stem cells accompanying the implant could address the problem of remodeling. The administration of hydrogel material serves as a two pronged strategy; first, to act as a support matrix to the heart and prevent any remodeling due to the infarct; and second, to allow retention of cells administered within it, further improving LVEF. Furthermore, the hydrogel must be able to degrade over time and allow cells to take over the supporting role after tissue regrowth [208]. Although the regulatory hurdles and the translational challenges in just administration of hydrogels materials are immense, making the journey of hydrogel material scaffold along with cells and growth factors a strategy with long-term fruition [195].

Executive summary

Stem cells for cell therapy

- Stem cells are available with various levels of benefits for an infarct condition.
- Pluripotent stem cells offer the better solution in terms of the final cell population that can be derived and applied.

Clinical studies

- Clinical studies have reported safety, efficacy and dosage response to stem cell populations. Cardiac stem cells and mesenchymal stem cells offer by far the best alternatives for auto and allogenic transfer. Pluripotent stem cells are yet to be evaluated clinically.

Conclusion

- Stem cell and biomaterial approaches are being investigated separately under clinical conditions. Furthermore, small molecule delivery for differentiation is still not under consideration.

Future perspective

- The dosage, delivery and integration will have to be an approach where, biomaterials act as carriers, support for the heart and matrix for differentiation, small molecules aid differentiation and cells compensate for the loss.

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REVIEW

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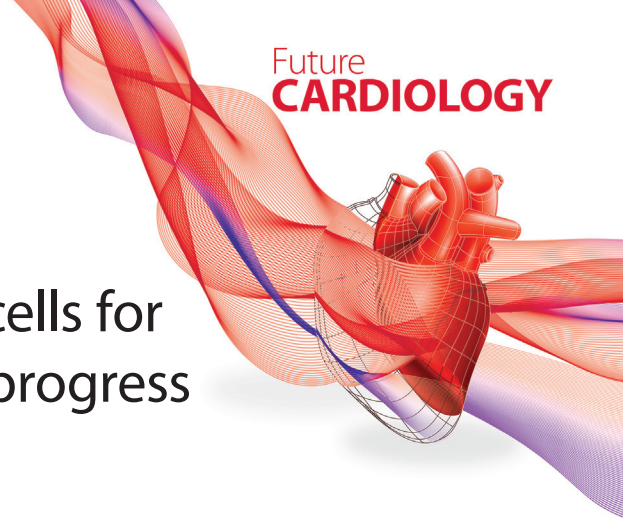

Allogeneic cardiosphere-derived cells for myocardial regeneration: current progress and recent results

Chris J Kapelios[1], John N Nanas[1] & Konstantinos Malliaras*[,1]

Early-phase clinical testing of autologous cardiosphere-derived cells (CDCs) has yielded intriguing results, consistent with therapeutic myocardial regeneration. However, autologous therapy is associated with significant technical, timing, economic and logistic constraints, prompting researchers to explore the potential of allogeneic CDC therapy. CDCs exhibit a favorable immunologic antigenic profile and are hypoimmunogenic *in vitro*. Preclinical studies in immunologically mismatched animals demonstrate that allogeneic CDC transplantation without immunosuppression is safe and produces sustained functional and structural benefits through stimulation of endogenous regenerative pathways. Currently, allogeneic human CDCs are being tested clinically in the ALLSTAR and DYNAMIC trials. Potential establishment of clinical safety and efficacy of allogeneic CDCs combined with generation of highly standardized, 'off-the-shelf' allogeneic cellular products would facilitate broad clinical adoption of cell therapy.

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• allogeneic cells • cardiosphere-derived cells • cell therapy • heart regeneration • heart repair

Despite significant advances in pharmacological and device-based therapies, ischemic heart disease remains the number one cause of death and years of life lost in the world [1]. In an attempt to address the need for development of novel therapies, cell therapy has emerged over the past 15 years as a potential therapeutic approach for ischemic cardiomyopathy and heart failure [2]. Among the multiple cell types that have been tested (including cells isolated from skeletal muscle [3], the bone marrow [4,5] or the heart itself) [6,7] cardiosphere-derived cells (CDCs) [8] appear particularly attractive for myocardial repair and regeneration, given the intriguing results observed in animal studies and in the first-in-man CArdiosphere-Derived aUtologous stem CElls to reverse ventricULar dySfunction (CADUCEUS) trial [7,9]. The majority of studies of cell therapy to date have been conducted using autologous (or syngeneic) cells. However, autologous cell therapy is associated with significant limitations (described later), which have prompted researchers to explore the potential of allogeneic cell therapy [10–13]. In this review, we focus on allogeneic CDCs as potential therapeutic agents for heart disease. We describe the *in vitro* immunologic properties of CDCs (from the standpoint of allogeneic cell transplantation), summarize the available *in vivo* preclinical data on allogeneic CDCs, discuss ongoing clinical trials of allogeneic CDCs and touch upon the critical issue of development of potency assays for allogeneic cellular products. The focus of this review lies solely on allogeneic CDCs (or other heart-derived cells that appear similar to CDCs). General discussion of immunologic barriers to allogeneic cell transplantation or discussion of other allogeneic cell types (e.g., allogeneic mesenchymal stromal cells) is intentionally cursory. The reader is referred elsewhere for reviews on these topics [14–16].

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Mesenchymal stromal cells: the prototypical 'universal donor' cells for heart repair

Allogeneic mesenchymal stromal cells (MSCs) are considered the prototypical 'universal donor' cells for cardiac applications. MSCs exhibit an immunologic profile that renders them attractive for allogeneic transplantation without concurrent immunosuppression: they express major histocompatibility complex (MHC) class I, but lack expression of MHC class II surface antigens or costimulatory molecules. In addition, MSCs exert multiple immunosuppressive and immunomodulatory effects *in vitro*, as they have been shown to interact with virtually all immune cells involved in adaptive and innate immunity. More specifically, MSCs (through both paracrine and contact-dependent mechanisms) inhibit T-cell and B-cell proliferation, inhibit the maturation and cytotoxicity of natural killer (NK) cells, dampen the respiratory burst of neutrophils, inhibit the maturation of monocytes into dendritic cells and impair the antigen-presenting function of dendritic cells (see [15] for an excellent review). Preclinical studies in large animal models of ischemic cardiomyopathy have demonstrated that allogeneic MSC transplantation without immunosuppression is safe and produces benefits that appear equivalent to those produced by autologous cells [17]. With regard to clinical translation, early-phase clinical testing of allogeneic MSCs in patients with heart disease has yielded promising results; allogeneic human MSCs have demonstrated a favorable safety profile (without eliciting a significant immune memory response in transplanted recipients) and have produced encouraging hints of efficacy [10–12]. It should be noted that even immunoprivileged cells like MSCs are eventually rejected after *in vivo* transplantation without concurrent immunosuppression [18–21]; however, since their mechanism of action is indirect [22], rejection of MSCs may not be of therapeutic significance if it is delayed long enough to allow them to exert their reparative paracrine effects.

Properties of CDCs & clinical translation of autologous CDC therapy

Over the past decade, CDC therapy has navigated the entire trajectory from discovery through early-stage clinical trials [23]. Unlike many other cell types that have reached the clinic, CDCs followed a systematic preclinical developmental program, comprising *in vitro* studies (to optimize cell culture methods), small animal studies (proof-of-concept studies) and large animal studies (to optimize dosage, formulation and delivery), before moving on to human subjects in the first-in-man CADUCEUS trial. After reviewing the accumulated preclinical data in their totality, we herein attempt to summarize the most important properties of CDCs.

CDCs are cultured as follows: in brief, myocardial specimens (obtained from percutaneous biopsies, surgical biopsies or explanted donor hearts) are minced and plated in primary culture to yield cardiac outgrowth cells. Outgrowth cells are then harvested and plated into suspension culture, in order to generate spherical multicellular clusters termed cardiospheres (CSps) [24]. CSps provide a microenvironment that favors upregulation of stemness and cell–matrix interactions [24,25]. CSps are collected, replated and further expanded in monolayer culture to yield CDCs [8]. CDCs are cells of intrinsic-cardiac origin [26]. Surface markers are uniformly positive for endoglin (CD105, the TGF-β receptor subunit) and uniformly negative for the pan-hematopoietic marker CD45 [8]. A fraction of CDCs (25–60%) expresses CD90 [27–29] (a marker of MSCs), and a small minority (~3%) expresses c-Kit (a putative marker of endogenous cardiac progenitors) [27]. Importantly, it has been demonstrated that the $CD105^+$/$CD90^-$/$c\text{-}Kit^-$ population of CDCs constitutes the active fraction, in terms of therapeutic efficacy; the $c\text{-}Kit^+$ fraction and the $CD90^+$ fraction are neither necessary for, nor contributory to, the regenerative efficacy of CDCs [27]. CDCs do not express markers typically associated with fibroblasts or myofibroblasts (<4% of CDCs express discoidin domain-containing receptor 2 or smooth muscle actin) [30] and, at least in their naive state, CDCs are generally negative for sarcomeric proteins [27]. Autologous CDCs can be isolated from percutaneous endomyocardial biopsy specimens and can be readily expanded in culture [8], yielding clinically relevant numbers in a timely manner [7]. Several animal studies from multiple independent laboratories have demonstrated that CDCs improve cardiac function, decrease scar size and increase viable myocardium postmyocardial infarction (MI) in mice [8,31–32], rats [30,33–36] and pigs [37–41]. While the therapeutic superiority of CDCs over other adult progenitor cells has not been established, preliminary comparative studies suggest that CDCs appear to be more potent than [32,37]

(or at least equally effective as [22]) other clinically applicable cell types. Importantly, from a translational perspective, dose-ranging studies in clinically relevant large animal models of ischemic cardiomyopathy have demonstrated that intracoronary infusion of CDCs (up to 300,000 CDCs/kg of body weight) does not cause microvascular obstruction and cardiac injury [38].

As far as the mechanism of action underlying CDC-induced myocardial repair goes, it is now evident that indirect mechanisms trump stemness; even though CDCs are clonogenic, self-renewing and multipotent [42], the salutary effects of CDC therapy should be attributed to indirect mechanisms of action, rather than direct cardiomyogenic differentiation of administered cells [30,31]. Accordingly, it has been shown that CDCs, both through paracrine and contact-dependent mechanisms, promote endogenous repair and regeneration by stimulating angiogenesis, recruitment of endogenous progenitors and proliferation of resident cardiomyocytes in the peri-infarct area [30–31,43–44]. While the mediators of these regenerative effects have not been fully elucidated yet, fate-mapping studies have demonstrated that CDCs stimulate recruitment of endogenous progenitors partially through secretion of SDF-1. In addition, CDCs are potent secretors of exosomes (rich in miRNAs), which appear to be critical agents of CDC-induced regeneration, angiogenesis and cardioprotection [45–47]. Among the several miRNAs enriched in CDC-derived exosomes, miR-146a, miR-210 and miR-132 appear particularly intriguing; preliminary mechanistic studies have demonstrated a cardioprotective and regenerative effect of miR-146a [46,47], an antiapoptotic effect of miR-210 [46] and a proangiogenic effect of miR-132 [46].

With regard to clinical translation, two clinical trials of autologous CDCs (the CADUCEUS trial and the TICAP trial) have been completed [7,9,29]. The Phase I CADUCEUS trial (performed in the Cedars-Sinai Heart Institute, CA, USA and the Johns Hopkins Hospital, MD, USA) investigated the feasibility, safety and preliminary efficacy of intracoronary infusion of autologous CDCs in patients with convalescent MI [7,9]. Thirty-one patients with subacute MI and left ventricular dysfunction were randomized in a 2:1 manner to receive either intracoronary infusion of 12.5–25 million autologous CDCs (grown from endomyocardial biopsies) or standard care and were followed over a 1-year period. Twenty-five patients (17 treated, 8 controls) were included in the per-protocol analysis. Autologous CDCs were successfully harvested from endomyocardial biopsies and grown in sufficient numbers to achieve the required CDC dose in 17/20 patients; three technical manufacturing failures occurred. Intracoronary infusion of autologous CDCs in the infarct-related artery did not raise significant safety concerns. Analysis of exploratory efficacy endpoints, measured by cardiac MRI, revealed a decrease in scar size, an increase in viable myocardium and improved regional contractility of infarcted myocardium (but not ejection fraction) in CDC-treated patients compared to controls. While the lack of increased benefit in ejection fraction after CDC therapy may appear paradoxical at first (ejection fraction improved similarly – by ~6% – in CDC-treated and control patients), it needs to be highlighted that ejection fraction is dependent on several parameters (including volume load, ventricular geometry and electrical activation pattern) and therefore may not be the best-suited surrogate marker for evaluating efficacy of cell therapy [23]. Importantly, the CDC-induced decrease in scar size was consistent with the increase in ejection fraction observed in cell-treated patients, as predicted by the natural relationship between scar size and ejection fraction in convalescent MI [9]. In addition, scar shrinkage correlated with an increase in viable myocardium [7,9] and with improvement in regional function [9], suggesting genuine therapeutic myocardial regeneration. While the fidelity of contrast-enhanced cardiac MRI in assessing tissue viability after cell therapy has recently been called into question [48,49], studies in large animal models of convalescent MI have demonstrated that cardiac MRI can accurately measure scarred and viable myocardium after cell administration and thus can be reliably used for assessing regenerative efficacy of cell therapy [40,50].

The Phase I TICAP trial (Transcoronary Infusion of Cardiac Progenitor Cells in Patients with Single Ventricle Physiology, performed in Okayama University Hospital, Japan) investigated the feasibility, safety and preliminary efficacy of global intracoronary infusion (i.e., infusion in all three major coronary arteries) of autologous CDCs in pediatric patients with hypoplastic left heart syndrome [29]. Eighteen patients (~2 years old) with hypoplastic left heart

syndrome were prospectively assigned to receive global intracoronary infusion of autologous CDCs (300,000/kg of body weight) after staged palliation (n = 10), followed by eight controls with staged palliation alone. Fourteen patients (seven treated, seven controls), followed over an 18-month period, were included in the per-protocol analysis. Autologous CDCs were successfully grown in 9/10 patients; one technical manufacturing failure occurred. Intracoronary infusion of CDCs into all three major coronary arteries did not raise significant safety concerns. In addition, hints of efficacy were observed; pediatric patients treated with autologous CDCs exhibited improved ventricular function (measured by cardiac MRI), reduced heart failure symptoms and improved somatic growth compared with controls. The preliminary efficacy signals observed in these early-Phase trials of CDC therapy need to be tested in larger clinical trials.

Limitations of autologous cell therapy

Most clinical trials of cell therapy to-date have been conducted using autologous cells. While autologous cell therapy carries no risk of immune rejection, it requires patient-specific tissue harvesting, cell manufacturing and quality control and is, therefore, associated with important limitations. First, autologous tissue harvesting (via endomyocardial biopsies in the case of autologous CDCs) is invasive and not risk-free [51]. Second, culture of autologous cells may be complicated by technical manufacturing failures; in CADUCEUS, three such failures occurred: one bacterial contamination, one cytogenetic abnormality and one failure to achieve the minimal CDC dose for infusion [7]. Third, expansion of autologous heart-derived cells to clinically relevant numbers poses significant timing constraints (4–6 weeks for CDCs) [7], which prohibit administration of autologous heart-derived cellular products in acute MI or early post-MI. Fourth, donor age and comorbidities may [52] (or may not) [53] negatively impact upon cell quality, resulting in decreased cell potency. Finally, the technical, economic and logistic constraints associated with autologous cell therapy preclude broad adoption of cell therapy and limit its clinical application to few specialized centers.

The aforementioned limitations of autologous therapy could be overcome by the use of allogeneic cells. The obvious disadvantage of allogeneic therapy is the risk of immune rejection of transplanted cells, which could limit effectiveness of cell therapy (due to rapid rejection of transplanted cells), raise safety concerns (as it could theoretically result in immune-related myocardial injury), and induce allosensitization of the recipient, which (if robust and persistent) could complicate future organ transplant. However, if allogeneic cell therapy were proven to be safe and effective, it would open up a new paradigm in cellular therapeutics. Large-scale expansion of cells derived from allogeneic tissues could be performed in specialized manufacturing labs under strict quality control. In the case of heart-derived cells, attractive sources of allogeneic tissue include hearts explanted from organ donors but not used for transplantation and surgical myocardial discards. Highly standardized, 'off-the-shelf' allogeneic cellular products would subsequently be shipped to hospitals throughout the world and banked for future use, thus enabling broad adoption and timely application of cell therapy in a cost-efficient manner. This attractive novel paradigm has motivated researchers to investigate the safety and efficacy of allogeneic therapy for myocardial repair.

Figure 1 depicts schematically the preclinical developmental program and the clinical translation of allogeneic CDCs. The major findings of each step along the pathway to clinical translation are presented.

In vitro immunologic properties of heart-derived cells

Malliaras *et al.* investigated the *in vitro* immunologic properties of allogeneic rat and human CDCs [30]. CDCs were shown to express MHC class I, but not MHC class II surface antigens or CD80/CD86 costimulatory molecules. This antigenic profile appears favorable for allogeneic applications, since expression of MHC class I antigens would protect donor CDCs from recipient NK cells [54]; lack of expression of MHC class II antigens would allow donor CDCs to escape direct recognition by recipient CD4 T-helper cells; and the lack of costimulatory molecules on the surface of donor CDCs would prevent activation of recipient effector T cells by MHC class I surface antigens [55]. Mixed lymphocyte reactions demonstrated that allogeneic CDCs exhibit minimal *in vitro* immunogenicity, manifested as negligible lymphocyte proliferation and pro-inflammatory cytokine secretion.

Lauden *et al.* performed an in-depth investigation of the *in vitro* immunologic properties of a different (at first glance) type of heart-derived

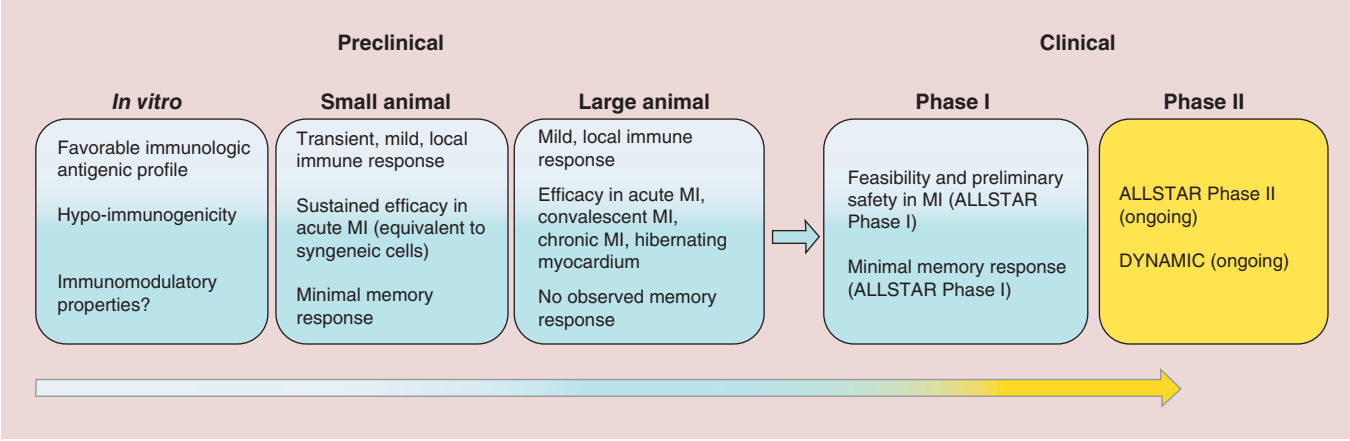

Figure 1. Preclinical developmental program and clinical translation of allogeneic cardiosphere-derived cells. Allogeneic cardiosphere-derived cells followed a systematic preclinical developmental program, comprising *in vitro* studies (to investigate immunological properties), small animal studies (proof-of-concept studies) and large animal studies (to investigate safety and efficacy in clinically relevant models), before moving on to human subjects in the first-in-man ALLSTAR and DYNAMIC clinical trials. The major findings of each step along the pathway to clinical translation are presented. Light blue denotes completed steps, while yellow denotes ongoing investigations.
MI: Myocardial infarction.

cells (termed 'human cardiac progenitor cells' [hCPCs] by the authors) [56]. hCPCs were grown as follows: c-Kit$^+$/CD45$^-$ cells were isolated from human surgical discards and were subsequently expanded in culture. Of note, the isolated heart-derived cells lost expression of c-Kit antigen during *ex vivo* expansion, resulting in generation of a cell population (hCPCs) that exhibited an antigenic profile strikingly similar to that of CDCs (hCPCs were uniformly CD105$^+$/CD45$^-$ and only approximately 4% expressed c-Kit). The loss of c-Kit expression during *ex vivo* expansion is in contrast to previous reports suggesting that c-Kit$^+$ cardiac cells demonstrate a relatively stable phenotype during long-term cell culture [57], and may be attributable to differences in cell culture methods. hCPCs, similar to CDCs, expressed MHC class I, but not MHC class II surface antigens. In addition, hCPCs were negative for costimulatory molecules CD40, CD80, CD86 and CD275, but expressed programmed death ligand 1 (PD-L1) (CD274), a costimulatory molecule of the B7 family that plays a critical role in regulation of T cell-mediated immune responses [58,59]. Mixed lymphocyte reactions demonstrated that allogeneic hCPCs were hypoimmunogenic and possessed immunosuppressive properties (i.e., they suppressed proliferation of preactivated lymphocytes). With regard to the latter, the authors demonstrated that the immunomodulatory capacity of hCPCs was mediated by hCPC-induced expansion of suppressive CD4$^+$/CD25^{high}/CD127^{low}/FoxP3^{high} effector regulatory T cells, via cell–cell contact-dependent interaction with PD-L1 (expressed on the surface of hCPCs). In a subsequent study from the same lab, Boukouaci *et al.* investigated the interaction of hCPCs with NK cells [60], by coculturing hCPCs with mismatched-allogeneic NK cells in medium containing IFN-γ (in an attempt to mimic the inflammatory milieu of infarcted myocardium). The authors found that under inflammatory conditions, allogeneic hCPCs not only evade killing by NK cells, but also suppress NK-cell cytotoxicity and modulate NK-cell cytokine secretion toward an anti-inflammatory profile.

Taken together, multiple studies from independent laboratories have demonstrated that CD105/CD45$^-$/c-Kit$^-$ heart-derived cells are hypoimmunogenic (and possibly immunosuppressive) *in vitro*, and exhibit an immunologic profile that, at least theoretically, renders them attractive for allogeneic applications without requiring concomitant immunosuppression.

In vivo preclinical studies of allogeneic CDC transplantation

The favorable *in vitro* immunological profile of CDCs led to the design of animal studies exploring the potential of allogeneic CDC therapy *in vivo* (summarized in **Table 1**). Malliaras *et al.* investigated the safety and efficacy of allogeneic CDC therapy (without concomitant

immunosuppression) in infarcted rats [30]. In order to create a stringent model of allogeneic cell transplantation, inbred rats from two immunologically divergent strains characterized by complete mismatch of MHC antigens were used as cell donors and recipients. Rats underwent MI and were randomized to receive syngeneic CDCs, allogeneic CDCs, xenogeneic CDCs (human CDCs, used as a positive control for immune rejection) or vehicle in the infarct border zone. Quantification of cell engraftment revealed that syngeneic and allogeneic CDCs survived at similar levels in infarcted recipient myocardium one week after delivery, but few syngeneic and even fewer allogeneic CDCs remained at three weeks post-cell administration. Allogeneic CDCs induced a mild, local immune response that had completely subsided by six months postdelivery and was not associated with any immune-related myocardial damage. Allogeneic CDCs did not induce systemic immunogenicity, as levels of circulating proinflammatory (IFN-γ, TNF-α, IL-1b, KC/GRO) and anti-inflammatory (IL-5, IL-13, IL-4) cytokines were comparable in rats receiving syngeneic or allogeneic CDCs. With regard

Table 1. Preclinical studies of allogeneic cardiosphere-derived cells.

Study (year)	Model	Route	Cells	Immune response	Efficacy	Ref.
Malliaras *et al.* (2012)	Rat, acute MI	IM	CDCs	Mild/transient local mononuclear infiltration, no DSAs, plus cellular memory response	↓scar size, ↑function, ↑myocyte cycling, ↑angiogenesis Equal benefits of allogeneic and syngeneic CDCs	[30]
Tseliou *et al.* (2013)	Rat, acute MI	IM	CSps	No local immune response	↓scar size, ↑function, ↑angiogenesis Equal benefits of allogeneic and syngeneic CSps	[61]
Malliaras *et al.* (2013)	Pig, convalescent MI	IC	CDCs	Mild/transient local mononuclear infiltration, no DSAs	↓scar size, ↓scar mass, ↑viable mass, ↑ (global, regional) function, ↓volumes, ↑myocyte cycling, ↑progenitors, ↑angiogenesis	[40]
Yee *et al.* (2014)	Pig, chronic MI	IM	CSps	Mild/transient local mononuclear infiltration, no DSAs	↓scar size, ↑viable mass, – global function, ↓LV volumes	[62]
Gallet *et al.* (2015)	Pig, convalescent MI	IC	CSps	Mild/ transient local mononuclear infiltration, no DSAs	↓scar size, ↓scar mass, ↑viable mass, ↑ (global, regional) function, ↓volumes, ↑perfusion, ↑angiogenesis	[63]
Suzuki *et al.* (2014)	Pig, hibernating myocardium	IC	CDCs	Not assessed	↑ (global, regional) function, ↑myocyte number, ↑myocyte cycling	[41]
Weil *et al.* (2015)	Pig, hibernating myocardium	IC	CDCs vs MSCs	Not assessed	↑ (global, regional) function, ↑myocyte number, ↑angiogenesis, ↑myocyte cycling Equal benefits of CDCs and MSCs, except for ↑myocyte cycling with CDCs	[22]
Kanazawa *et al.* (2015)	Pig, acute MI	IC	CDCs	Not assessed	↓scar size, ↓'no reflow' area, – global function, – LV volumes, ↓ apoptosis	[64]
Crisostomo *et al.* (2015)	Pig, acute MI vs convalescent MI	IC	CPCs	Not assessed	– size, – on global function, ↑viability within risk area, ↓ volumes (only in convalescent MI), ↑angiogenesis (only in acute MI)	[65]

↑ Increase; ↓ Decrease; – No effect; CDC: Cardiosphere-derived cell; CPC: Cardiac progenitor cell; CSp: Cardiosphere; DSA: Donor-specific antibodies; IC: Intracoronary; IM: Intramyocardial; LV: Left ventricle; MI: Myocardial infarction; MSCs: Mesenchymal stromal cells.

to development of immune memory response, no alloreactive circulating antibodies (donor-specific antibodies [DSAs]) were detected in recipients of allogeneic CDCs (suggesting no development of humoral memory response). However, mixed lymphocyte reactions demonstrated that lymphocytes isolated from recipients of allogeneic CDCs exhibited increased proliferation when cocultured with allogeneic CDCs, suggesting development of cellular memory response after allogeneic CDC therapy; whether the intensity of the memory response diminishes with time (as reported in studies of allogeneic MSCs) [20] and whether the observed memory response would result in accelerated rejection of a repeat dose of the same batch of allogeneic cells *in vivo* are issues that remain to be investigated. Importantly from a therapeutic standpoint, allogeneic and syngeneic CDCs produced indistinguishable benefits in cardiac structure (increase in viable myocardium, decrease in scar tissue) and function (improved systolic performance), which persisted for at least 6 months post-cell delivery (long after allogeneic cells had been cleared from the recipient myocardium). In addition, allogeneic CDCs stimulated endogenous reparative mechanisms (cardiomyocyte cycling, angiogenesis) and increased myocardial levels of VEGF, IGF-1 and HGF equally with syngeneic CDCs. In a subsequent study from the same lab, Tseliou *et al.* investigated the safety and efficacy of intramyocardial injection of allogeneic CSps, without concomitant immunosuppression, in immunologically mismatched infarcted rats [61]. Allogeneic CSps did not elicit a significant local immune response *in vivo* and exhibited similar levels of cell engraftment as syngeneic CSps at 1 and 3 weeks postdelivery. Importantly, allogeneic and syngeneic CSps produced similar durable functional and structural benefits post-MI, and promoted comparable upregulation of beneficial paracrine factors (VEGF, IGF-1 and HGF) in the peri-infarct area.

The encouraging results obtained from the aforementioned small animal studies prompted the investigation of allogeneic CDC therapy in clinically relevant large animal models of MI and chronic ischemic cardiomyopathy. Malliaras *et al.* investigated the safety and efficacy of intracoronary infusion of allogeneic CDCs (without concomitant immunosuppression) in immunologically mismatched pigs with convalescent MI [40]. Yucatan minipigs underwent induction of MI and 2–3 weeks later were randomized to receive intracoronary infusion of mismatched allogeneic CDCs or vehicle in the infarct-related artery. Allogeneic CDCs induced a mild local lymphoplasmacytic infiltration in interstitial and perivascular spaces of the peri-infarct area, but did not result in any rejection-related myocardial damage. With regard to development of immune memory response, no circulating alloreactive antibodies (DSAs) were detected in recipients of allogeneic CDCs. In terms of efficacy, allogeneic CDCs produced significant structural benefits (increase in viable myocardium and decrease in scar mass), improved global and regional cardiac function and stimulated endogenous regenerative mechanisms (cardiomyocyte cycling, recruitment of endogenous cardiac progenitors, angiogenesis). Importantly, histological analysis of explanted hearts ruled out myocyte hypertrophy as a contributor to the increase in viable myocardium observed after allogeneic CDC therapy; myocyte size was actually smaller in the risk region of CDC-treated animals compared with controls, a finding consistent with cardiomyocyte hyperplasia and attenuation of remodeling-associated cardiomyocytes after cell therapy.

Yee *et al.* investigated the safety and efficacy of allogeneic CSps delivered by percutaneous NOGA-guided injections without concomitant immunosuppression, in immunologically mismatched pigs with chronic ischemic cardiomyopathy [62]. First, the investigators performed a dose-ranging study in infarcted minipigs, in order to optimize CSp dosage. The superior CSp dose (150 million CSp-forming cells, based on the results of the dose-ranging study) was then tested in a subsequent pivotal study; Yucatan minipigs underwent MI creation and 8 weeks later were randomized to receive transendocardial dose-optimized injection of allogeneic CSps or vehicle in the peri-infarct area. Allogeneic CSps exhibited a favorable safety profile *in vivo*; no excess myocardial inflammation and no development of alloreactive antibodies (DSAs) were observed in treated animals. In terms of efficacy, cardiac MRI revealed that transendocardial injection of allogeneic CSps decreased scar size, increased viable mass and attenuated left ventricular dilatation (but had no effect on global systolic function).

In a recent study, Gallet *et al.* investigated the safety and efficacy of intracoronary infusion of allogeneic CSps in pigs with convalescent MI [63]. Yucatan minipigs underwent induction

of MI and 1 month later were randomized to receive dose-optimized intracoronary infusion of allogeneic CSps (without concomitant immunosuppression) or vehicle in the infarct-related artery. Allogeneic CSps induced a mild local mononuclear infiltration in the peri-infarct area, but did not result in any rejection-related myocardial damage or in robust DSAs. Cardiac MRI revealed that intracoronary infusion of allogeneic CSps decreased scar size, increased viable mass, attenuated remodeling, improved global and regional myocardial function, and increased myocardial perfusion (measured both by MRI and by coronary flow reserve) compared to the control group.

In a study from the Canty lab, Suzuki *et al.* investigated the efficacy of global intracoronary infusion of allogeneic CDCs, with concomitant cyclosporine immunosuppression, in a porcine model of hibernating myocardium (from chronic occlusion of the left anterior descending artery) [41]. Allogeneic CDCs were infused in all three major coronary arteries, as the researchers aimed to target both the hibernating and the remote remodeled myocardium. Global intracoronary infusion of allogeneic CDCs did not raise safety concerns and improved global and regional cardiac function over a 4-week follow-up period. In agreement with previous studies [30,40,43], CDC administration stimulated resident myocyte cycling, increased myocyte nuclear density and reduced myocyte size; these findings are consistent with CDC-induced upregulation of resident myocyte proliferation resulting in generation of new smaller myocytes. Recently, the same group performed a head-to-head comparison of global intracoronary infusion of allogeneic bone marrow-derived MSCs versus allogeneic CDCs (with concomitant cyclosporine immunosuppression) in the aforementioned porcine model of hibernating myocardium. Both allogeneic MSCs and CDCs improved regional cardiac function, decreased myocyte size and increased myocyte density to the same extent compared with control animals over 4 weeks of follow-up. Allogeneic CDCs were superior to allogeneic MSCs in promoting cycling of resident cardiomyocytes, as manifested by a twofold increase in the number of $Ki67^+$ and $H3P^+$ myocytes in CDC-treated hearts compared to MSC-treated hearts [22].

As discussed earlier in this review, a major advantage of allogeneic cell therapy is that it is not bound by the timing constraints of autologous cell therapy (as the former does not necessitate patient-specific tissue harvesting and cell processing). Therefore, the use of allogeneic 'off-the-shelf' cellular products enables timely application of cell therapy in the acute MI setting. In patients with acute MI, intracoronary administration of allogeneic cells immediately after coronary reperfusion appears particularly attractive both from a practical standpoint (it obviates the need for a second catheterization procedure for cell infusion) as well as from a cardioprotective standpoint (since most cardiomyocytes die during the first 24 h post-MI [66], interventions beyond this timeframe have minimal potential for myocardial salvage). A caveat of this therapeutic approach is aggravation of microvascular obstruction (which occurs naturally after reperfusion in acute MI) via coronary microembolization of administered cells [67]. Kanazawa *et al.* investigated the safety and efficacy of intracoronary infusion of allogeneic CDCs in pigs with acute MI [64]. Yucatan minipigs underwent MI and were randomized to receive intracoronary infusion of allogeneic CDCs or vehicle solution 30 min postreperfusion. Dose-optimized infusion of allogeneic CDCs was well-tolerated in the setting of acute MI (no significant changes in TIMI flow post-cell infusion were observed). Histology of infarcted hearts (explanted 48 h post-MI) demonstrated that infusion of allogeneic CDCs decreased infarct size, microvascular obstruction (i.e., 'no reflow' area, measured after thioflavin dye administration) and cardiomyocyte apoptosis in the risk region compared with control animals. The fact that structural benefits were detected this early (48 h post-cell delivery) suggests that the decreased infarct size observed in treated hearts should be attributed to an acute cardioprotective effect conferred by allogeneic CDCs rather than to CDC-induced myocardial regeneration. Whether this acute cardioprotective effect translates into longer-term benefits remains to be investigated.

Crisostomo *et al.* investigated the optimal timing of allogeneic heart-derived cell therapy early post-MI, by comparing delivery of allogeneic cells (without concomitant immunosuppression) in the acute MI setting versus 1 week post-MI [65]. Intracoronary infusion of allogeneic CPCs (either postreperfusion or 1 week post-MI) did not raise significant safety concerns. In terms of efficacy, both cell-treated groups exhibited attenuation of remodeling (as assessed by cardiac MRI), increased amount of

viable myocardium within the risk region and more mature vessels in the infarct border zone compared with control animal. Timing of cell delivery did not seem to significantly affect the therapeutic efficacy of the administered allogeneic CPCs, except for a trend toward greater attenuation of left ventricle dilatation observed in animals treated at 1 week post-MI compared with those treated immediately post-MI.

Taken together, preclinical studies have demonstrated that allogeneic CDC transplantation without immunosuppression is safe and produces durable structural and functional benefits, indistinguishable from syngeneic transplantation, in animal models of ischemic cardiomyopathy. The persistence of benefit, despite ephemeral cell survival, is rationalized by the indirect mechanism of action of transplanted cells [30,31]. It appears that allogeneic CDCs, possibly due to their hypoimmunogenic profile, can survive in recipient myocardium long enough to stimulate endogenous regenerative and reparative pathways, resulting in sustained benefit without the requirement for stable engraftment of transplanted cells.

Clinical studies of allogeneic cardiosphere-derived cells transplantation

The preclinical work described above motivated the testing of allogeneic human CDCs as potential 'off-the-shelf' therapeutic agents for heart disease. Two studies of allogeneic CDC therapy are currently underway, the ALLogeneic heart STem cells to Achieve myocardial Regeneration (ALLSTAR) [68] trial and the Dilated cardiomYopathy iNtervention with Allogeneic MyocardIally-regenerative Cells (DYNAMIC) trial [69].

ALLSTAR is a Phase I/II trial designed to investigate the feasibility, safety and efficacy of intracoronary infusion of allogeneic CDCs in patients with anterior MI (within the prior 1 year) and substantial scar burden (scar size: >15% of the left ventricle). In the recently completed open-label Phase I portion of the study (aiming at assessing feasibility and preliminary safety), 14 patients (left ventricular ejection fraction [LVEF]: ~42%, infarct size: ~25%) underwent intracoronary infusion of 12.5 million or 25 million allogeneic CDCs and were followed over a 1-year period [70]. Intracoronary infusion of allogeneic CDCs did not raise safety concerns; no prespecified primary safety endpoint (acute myocarditis, major adverse cardiac event, death due to arrhythmias or sudden unexpected death) occurred. With regard to development of immune memory response against the transplanted cells, four patients were found to have pre-existing DSAs against donor CDCs prior to infusion (one resolved and three persisted during follow-up). *De novo* DSAs developed in four subjects (three resolved and one persisted during follow-up), while no *de novo* cellular immune memory responses were detected. In terms of efficacy, cardiac MRI revealed that patients who received 25 million cells to which they had no pre-existing DSAs (n = 8) exhibited a significant decrease in infarct size (15% relative reduction) and a trend toward improvement in ejection LVEF (by ~4%) over the 1-year follow-up period. On the basis of the encouraging Phase I findings, ALLSTAR has proceeded to the randomized, double-blind Phase II portion of the trial, aiming at assessing safety and efficacy. In this portion of the study (which is currently recruiting), 260 patients without pre-existing DSAs against donor CDCs will be randomized (in a 2:1 fashion) to undergo intracoronary infusion of 25 million allogeneic CDCs or placebo solution and will be followed over a 1 year period. Two strata will be enrolled: recent MI, defined as index MI 28–90 days prior to treatment (n = 130), and chronic MI, defined as index MI 90–365 days prior to treatment (n = 130). Comparisons between the two strata may provide insight into the optimal timing of cell administration post-MI. The primary efficacy endpoint of the Phase II portion of ALLSTAR is infarct size reduction, as measured by MRI.

DYNAMIC is a Phase II trial designed to investigate the feasibility and safety of intracoronary infusion of allogeneic CDCs in patients with ischemic or non-ischemic dilated cardiomyopathy, systolic dysfunction (LVEF: <35%) and advanced heart failure (NYHA III/IVa). Patients with advanced heart failure may represent a more fertile target for cell therapy trials [71] since: they have significantly greater room for improvement compared with patients with MI; and results from several clinical trials to-date indicate that sicker patients are the ones who benefit the most from cell therapy [4,72–74]. In the open-label Phase IIa portion of DYNAMIC (which is currently in follow-up), 14 heart failure patients underwent intracoronary infusion (in all three major coronary arteries) of allogeneic CDCs; there have been no safety concerns to-date, and the patients will be followed

over a 1-year period. A placebo-controlled Phase IIb portion of DYNAMIC is anticipated. Prespecified primary safety endpoints of DYNAMIC include decreased TIMI flow post-cell infusion, acute myocarditis, major adverse cardiac events, death due to arrhythmias or sudden unexpected death. Patients are monitored for development of immune memory response against the transplanted allogeneic cells; this is of particular interest, as development of robust and persistent immune memory responses in patients with advanced heart failure could potentially complicate future heart transplantation. Functional testing (to gauge efficacy) includes cardiac imaging by computed tomography.

Potency assays

A critical step in the clinical translation of cell therapy, as mandated by regulatory authorities, [75] is development of relevant potency assays (i.e., tests to measure potency of manufactured cellular products). This is particularly important for allogeneic cell therapy, as development of appropriate potency assays would enable prospective identification of donor cells with greater potential for clinical efficacy. However, the fact that cell therapy products appear to have complex, multifactorial and not fully characterized mechanisms of action presents difficulties in establishing which cellular attributes are most relevant to measuring potency. Given that potency assays should ideally represent the cellular product's mechanism of action; [75] and multiple lines of evidence suggest that the mechanism of action of adult cells is indirect (i.e., is mediated by paracrine mechanisms) [30,31], it appears logical that *in vitro* biological assays measuring paracrine factor secretion may serve as appropriate potency assays for cellular products. CDCs, in particular, are potent secretors of several reparative cytokines [76] and miRNAs, [46,47] all of which (alone or in any combination) could potentially underlie the salutary effects of CDC therapy. Out of all identified paracrine factors, *in vitro* secreted SDF-1 by CDCs appears to be associated with *in vivo* potency, since: a) retrospective analysis of the properties of human CDCs administered in CADUCEUS revealed a positive correlation between *in vitro* SDF-1 secretion by CDCs and *in vivo* therapeutic outcome; [53] b) human CDCs that secrete higher levels of SDF-1 *in vitro* exhibit superior functional potency when injected in infarcted immunocompromised mice; [53] and c) mechanistic studies in fate-mapped mice demonstrate that CDCs amplify innate regeneration by endogenous progenitors, in part through the secretion of SDF-1 by transplanted cells [44]. Based on the above, *in vitro* SDF-1 secretion by CDCs merits prospective testing as a predictor of *in vivo* therapeutic efficacy. However, it must be noted that it is highly unlikely that the totality of CDC-induced benefits is mediated through SDF-1 secretion alone. Other potentially relevant potency assays of allogeneic CDCs include: a) surface expression of CD90 (as increased CD90 expression has been associated with decreased potency both in small animal studies and in CADUCEUS [27]); b) exosome secretion capacity (as exosomes appear to be critical agents of CDC-induced cardioprotection, regeneration and angiogenesis) [45–47]; c) miRNA secretion capacity (with miR-146a [46,47], miR-210 [46] and miR-132 [46] emerging as potentially attractive candidates); d) surface expression of PD-L1 (as PD-L1 appears to play a critical role in the immunosuppressive/immunomodulatory effects of CDCs [56]; therefore, allogeneic CDCs with increased expression of PD-L1 could potentially evade immune rejection more efficiently); and e) therapeutic efficacy in small animal models (i.e., administering human CDCs derived from different donors in immunodeficient animal models and selecting the ones that produce the greatest structural/functional benefits in animals for human applications). In any case, the clinical data collected from ongoing trials of allogeneic CDCs in conjunction with extensive *in vitro* testing of the administered allogeneic cell products will likely help better define which cellular attributes are predictors of greater clinical efficacy (and should therefore be measured as indicators of cell potency).

Conclusion & future perspective

During the next few years, significant insight is expected to be gained regarding the safety and efficacy of allogeneic CDCs both from preclinical studies (currently underway in multiple laboratories worldwide) and, particularly, from ongoing clinical investigations of allogeneic CDC therapy. Results from ongoing trials should be available within 1–2 years and will help answer pivotal questions regarding allogeneic CDC therapy: is intracoronary infusion of allogeneic CDCs safe and effective in patients with MI (ALLSTAR) or advanced heart failure (DYNAMIC)? Do allogeneic CDCs induce

humoral or cellular immune memory responses in human recipients? If so, are said immune responses transient or persistent? This is particularly relevant to patients with advanced heart failure (DYNAMIC) as it could potentially complicate future heart transplantation. What is the optimal time of cell administration post-MI? Is application of allogeneic cells earlier in the remodeling process associated with increased therapeutic benefit (comparison of recent MI stratum versus chronic MI stratum in the ALLSTAR trial)? Does targeting a sicker patient population (as the one enrolled in DYNAMIC) maximize the potential of allogeneic therapy to produce clinically meaningful benefits, like reductions in mortality or rehospitalizations? Will the collected clinical data combined with the results obtained from *in vitro* testing of the administered allogeneic cell products help better define which cellular attributes are predictors of greater clinical efficacy? In addition, new basic studies are needed to better elucidate the mechanism of action of CDCs and to investigate whether large-scale *ex vivo* expansion negatively impacts upon their regenerative potential. The latter is of particular importance, as it has been suggested that serial passaging of MSCs alters their immunophenotype (increases expression of MHC II molecules) [77,78], attenuates their immunomodulatory/immunosuppressive properties, [79] decreases their stemness/multipotentiality [80,81] and attenuates secretion of paracrine factors [80].

In any case, potential establishment of clinical safety and efficacy of allogeneic CDCs in conjunction with generation of highly standardized, 'off-the-shelf' allogeneic cellular products would certainly catalyze broad clinical adoption of cell therapy.

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EXECUTIVE SUMMARY

- Autologous cardiosphere-derived cells (CDCs) for heart repair have yielded positive results in early-phase clinical testing (CADUCEUS and TICAP clinical trials).
- Autologous therapy is associated with significant technical, timing, economic and logistic constraints, which could be overcome by the use of allogeneic cells.
- CDCs exhibit a favorable immunologic antigenic profile and are hypoimmunogenic (and possibly immunosuppressive) *in vitro*.
- Allogeneic CDC transplantation without immunosuppression in immunologically mismatched recipient animals is safe and produces sustained functional and structural benefits through stimulation of endogenous regenerative pathways.
- Allogeneic human CDCs are currently being tested clinically in the ALLSTAR and DYNAMIC clinical trials.
- Potential establishment of clinical safety and efficacy of allogeneic CDCs combined with generation of highly standardized, 'off-the-shelf' allogeneic cellular products would facilitate broad clinical adoption of cell therapy.

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