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Pluripotent stem cell-derived mesenchymal stromal cells improve cardiac function and vascularity after myocardial infarction

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ABSTRACT

Background aims: Mesenchymal stromal cells (MSCs) have been shown to improve cardiac function after injury and are the subject of ongoing clinical trials. In this study, the authors tested the cardiac regenerative potential of an induced pluripotent stem cell-derived MSC (iPSC-MSC) population (Cymerus MSCs) in a rat model of myocardial ischemia-reperfusion (I/R). Furthermore, the authors compared this efficacy with bone marrow-derived MSCs (BM-MSCs), which are the predominant cell type in clinical trials.
Methods: Four days after myocardial I/R injury, rats were randomly assigned to (i) a Cymerus MSC group (n = 15), (ii) a BM-MSC group (n = 15) or (iii) a vehicle control group (n = 14). For cell-treated animals, a total of 5×10^6 cells were injected at three sites within the infarcted left ventricular (LV) wall.
Results: One month after cell transplantation, Cymerus MSCs improved LV function (assessed by echocardiography) compared with vehicle and BM-MSCs. Interestingly, Cymerus MSCs enhanced angiogenesis without sustained engraftment or significant impact on infarct scar size. Suggesting safety, Cymerus MSCs had no effect on inducible tachycardia or the ventricular scar heterogeneity that provides a substrate for cardiac re-entrant circuits.
Conclusions: The authors here demonstrate that intra-myocardial administration of iPSC-MSCs (Cymerus MSCs) provide better therapeutic effects compared with conventional BM-MSCs in a rodent model of myocardial I/R. Because of its manufacturing scalability, iPSC-MSC therapy offers an exciting opportunity for an "off-the-shelf" stem cell therapy for cardiac repair.

Introduction

Severe left ventricular (LV) dysfunction after myocardial infarction (MI) is an important predictor of clinical outcome and is associated with increased morbidity/mortality from heart failure (HF) and sudden cardiac death [1]. Although current therapies for MI have significantly improved patient survival, there remain a large proportion who suffer progressive HF. These patients would benefit from novel methods to fundamentally repair the damaged heart and restore cardiac function. Over the last two decades, substantial progress has been made in the field of stem cell therapy for cardiac repair, with research proceeding rapidly from pre-clinical models to clinical studies [2]. Autologous bone marrow (BM) mononuclear cells as cell therapy for HF have been investigated in multiple randomized and non-randomized trials over the last 20 years, with more recent trials not showing significant clinical benefit [3−12]. In contrast to autologous strategies, allogeneic cell therapy offers an efficient way to achieve immediate availability, thus avoiding the need for aspiration and tissue culture delays before BM aspiration [13]. As such, allogeneic BM-derived mesenchymal stromal cells (BM-MSCs) have recently emerged as the leading candidate for an "off-the-shelf" therapeutic agent. MSCs (also called

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mesenchymal stromal cells) are considered immune-privileged and can be expanded in quantities unattainable from an autologous source [14]. The POSEIDON clinical trial was the first to demonstrate that the rate of alloimmune reactions in patients receiving allogeneic MSCs in ischemic LV was low, suggesting that allogeneic MSC transplantation may be accomplished without the need for host immunosuppression [15]. Although it was not powered to show efficacy, this study showed that allogeneic immune MSC treatment provided beneficial effects in adverse LV remodeling in patients with ischemic cardiomyopathy, offering an off-the-shelf, readily available cell product. Multiple mechanisms have been proposed for the beneficial effects of MSCs, including multidirectional differentiation potential and paracrine factor secretion with anti-apoptotic, pro-angiogenic and immunomodulatory effects [14].

Despite rapid clinical translation and widespread enthusiasm, the isolation of BM-MSCs is cumbersome and dependent on a continual supply of healthy donors. Induced pluripotent stem cells (iPSCs), by contrast, have the capacity to indefinitely proliferate without losing pluripotency and to maintain normal karyotype [16]. Harnessing the expansion potential of iPSCs prior to differentiation enables the production of very large numbers of MSCs from a single iPSC line, thus affording manufacturing feasibility and scalability [17]. In fact, iPSC-derived MSCs (iPSC-MSCs) have already reached the clinical trial stage. Cynata Therapeutics Limited (Melbourne, Australia) has recently completed a phase 1 clinical trial of allogeneic mesenchymal angioblast-derived MSCs (Cymerus MSCs) [17,18] for steroid-resistant graft-versus-host disease (NCT02923375) demonstrating safety and a signal for efficacy, with phase 2 trials expected to commence in 2021.

Given the efficacy of BM-MSC cardiac cell therapy, the authors hypothesized that transplantation of iPSC-MSCs following MI would result in improved cardiac function, with therapeutic effects correlating with cellular paracrine secretion. The aim of this study was to assess the engraftment potential, safety and cardiac reparative efficacy of iPSC-MSCs after transplantation into the infarcted rat heart (benchmarked against BM-MSCs used in clinical trials).

Methods

Cell culture

Human BM-MSCs were cultured on standard tissue culture plates (TCPs) in the presence of Minimum Essential Medium alpha, 1% GlutaMAX 100×, 100 U/mL penicillin and 100 mg/mL streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) as well as 20% fetal bovine serum (Bovogen Biologicals Pty Ltd, Melbourne, Australia). Cymerus MSCs were provided by Cynata Therapeutics Limited and produced as previously described [18]. The process is highly efficient, yielding a homogeneous population of CD105+, CD73+, CD90+, CD43/45−, CD31− and HLA-DR− MSCs. Cymerus MSCs at passage five were used in this study based on previously published clinical studies showing that this passage level facilitates the production of a workable batch size while remaining below the limit of the cells' expansion potential [17].

Angiogenic cytokine array

Cells were detached from the TCPs using Accutase (Gibco, Carlsbad, CA, USA) and seeded at 5000 cells/cm^2 density on a 12-well plate in Dulbecco's Modified Eagle's Medium (DMEM) low glucose (Gibco) with 10% fetal bovine serum and penicillin/streptomycin and incubated at 37°C and 5% carbon dioxide for 2 days. Conditioned media from each group were then collected and used to perform the angiogenic cytokine array test. A human angiogenesis antibody array—membrane (20 targets) (ab134000; Abcam, Cambridge, UK) was used for the detection of cytokines secreted by cells in the culture media (conditioned media) following the manufacturer's instructions. Briefly, membranes were first blocked with blocking buffer and then incubated overnight with conditioned media at 4°C. After overnight incubation of first biotinylated antibody cocktail and then horseradish peroxidase-conjugated streptavidin, cytokines were detected using ChemiDoc (Bio-Rad Laboratories, Inc, Hercules, CA, USA) by exposing the membranes at 2.0-s chemiluminescence exposure. After imaging, samples were analyzed using the Gilles Carpentier protein array analyzer for ImageJ macro (https://imagej.nih.gov/ij/macros/toolsets/Protein%20Array%20Analyzer.txt).

Cell preparation for intra-myocardial cell injection

Cymerus MSCs were prepared from clinical-grade iPSCs and provided by Cynata Therapeutics Limited. Briefly, iPSCs were derived from CD34-enriched peripheral blood mononuclear cells using an episomal plasmid-based, transgene-free, viral-free and feeder layer-free reprogramming procedure prior to differentiating and expanding in culture [19]. Upon receipt, cells were stored in liquid nitrogen. Before cell injection, cryopreserved human Cymerus MSCs were thawed in a 37°C water bath and washed twice with Plasma-Lye A solution (Baxter Healthcare Corporation, Deerfield, IL, USA), an electrolyte solution known to be compatible with cells [20]. A total of 5×10^6 cells were then resuspended in 100 μL of Plasma-Lye solution. Cells were kept cold on ice until administration to animals. BM-MSCs were purchased from AllCells (Alameda, CA, USA). Cryopreserved BM-MSCs were washed twice in DMEM and resuspended at the same density as Cymerus MSC derivatives (5×10^6 cells per 100 μL).

MI induction and cell transplantation

All animal procedures were approved by the Western Sydney Local Health District Animal Care and Ethics Committee (protocol identifier, 4214.02.14). Studies were conducted in male nude rats (CBH-RNU, 8−12 weeks). For all procedures, animals were anesthetized with 2% isoflurane and 0.2 L/min oxygen, endotracheally intubated and mechanically ventilated. Details of the randomized main study design are shown in Figure 1. At day −4, animals were anesthetized and underwent thoracotomy. MI was induced by ligation of the left anterior descending artery for 60 min followed by reperfusion [21,22]. Successful infarction was determined by blanching of the myocardium distal to the coronary ligation. Four days after ischemia-
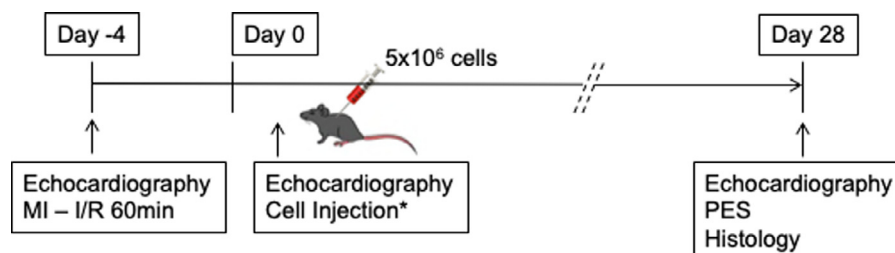
Figure 1. Main study timeline. Schematic diagram showing the overall animal study design used to test the therapeutic benefits of Cymerus MSCs in a rat model of myocardial I/R. I/R, ischemia-reperfusion. (Color version of figure is available online).

reperfusion (day 0), animals were randomly assigned to three groups: (i) MI + Plasma-Lye group (vehicle control, n = 14), (ii) MI + Cymerus MSC group (Cymerus MSCs, n = 15) and (iii) MI + BM-MSC group (BM-MSCs, n = 15). Animals underwent intra-myocardial injection of cells (5×10^6 in 100 μL Plasma-Lye) or vehicle controls (100 μL Plasma-Lye only) at three injection sites into infarct and border zones. Echocardiography and programmed electrical stimulation (PES) were conducted to assess for functional changes and arrhythmogenicity, respectively. Animals were killed on day 28, and hearts were excised for subsequent histological analysis.

Echocardiography

All rats underwent baseline echocardiography prior to commencement of experimental protocols. Echocardiographic studies were repeated 4 days post-MI (before cell injection) and 28 days following cell injection using a Philips ultrasound system (Philips Healthcare, Andover, MA, USA). Systolic function was measured by calculating the fractional shortening (FS) percentage derived from LV end-diastolic diameter and LV end-systolic diameter measurements in the parasternal short-axis view using M-mode tracings. All measurements were conducted by operators blinded to the treatment groups.

Programmed electrical stimulation

PES was performed at day 28 to assess animals at risk of sudden cardiac death. A 5Fr octopolar catheter (Biosense Webster Inc, Irvine, CA, USA) was placed transesophageally and advanced until ventricular electrogram was obtained. PES was performed using an electronic pulse stimulator (model 2100; A-M Systems, Sequim, WA, USA) based on a previously published PES protocol [23]. A drive train (S1S1) of eight beats at 180 ms was followed by four extrastimuli delivered one at a time. Initial extrastimuli were delivered at a coupling interval of 150 ms, which was then decremented by 10 ms until ventricular refractoriness. In the absence of ventricular tachycardia (VT) inducibility, a more rigorous protocol with burst ventricular pacing at cycle lengths of 90 ms and 60 ms was administered for 30 s. Sustained VT was defined as monomorphic or polymorphic wide complex tachycardia lasting greater than 10 s, induced by four or more extrastimuli or ventricular burst pacing, and was considered a positive result.

Morphometric and histological studies

Following euthanasia on day 28, infarcted hearts were harvested, fixed in 10% formalin, embedded in paraffin and then sectioned at 4-μm thickness for Gömöri trichrome staining. Images were digitally scanned using a NanoZoomer (Hamamatsu Photonics, Hamamatsu, Japan). Infarct size (as a percentage of LV) was analyzed using computerized planimetry as previously described [24].

Cell engraftment studies

Engraftment and survival of transplanted cells were analyzed at 1 week (non-infarcted hearts) and 1 month (infarcted hearts) post-cell injection by immunofluorescence using a mouse anti-human mitochondrial antibody at 1:1000 (ab92824; Abcam), allowing specific detection of cells of human origin.

Determination of capillary and arteriolar density

Capillary and arteriolar densities of heart sections immunostained with von Willebrand factor (vWF) and alpha smooth muscle actin (α-SMA), respectively, were assessed 28 days after cell injection. Capillaries were defined as vessels stained with vWF, and four or more 4′,6-diamidino-2-phenylindole-positive cells in the endothelium and arterioles were considered to be structures containing vWF

co-localized with α-SMA [21,25]. Sections were counterstained with 4′,6-diamidino-2-phenylindole. Five regions within the infarct, border and remote zones of each animal (n = 14 per group) were analyzed. Results were expressed as capillaries and arterioles per high-power field.

Statistical analysis

All data are expressed as mean ± standard error of the mean. Normal distribution was assessed using the Shapiro−Wilk test. One-way analysis of variance was used for analysis of multiple groups using SigmaPlot 12.5 software (Systat Software Inc, San Jose, CA, USA). Post-hoc analysis was performed with the Holm−Šídák test. $P < 0.05$ was considered statistically significant.

Results

Fate of transplanted Cymerus MSCs and BM-MSCs

To investigate the engraftment potential of Cymerus MSCs with direct comparison to BM-MSCs (n = 3 per group), the authors performed a pilot study, injecting 5×10^6 cells into the non-infarcted hearts of nude rats. One week following cell injection, transplanted human cells could be detected at the graft sites of Cymerus MSCs (Figure 2A,B). However, similar to BM-MSCs (data not shown), transplanted Cymerus MSCs were not evident at day 28, suggesting lack of survival and engraftment of transplanted cells.

Cymerus MSCs improve LV function 1 month post-MI

LV function was assessed by FS using echocardiography (Figure 3A) at day −4 (pre-infarct), day 0 (pre-cell injection) and day 28. FS was comparable at day 0 (Cymerus MSCs, 27.95 ± 4.9%, BM-MSCs, 27.29 ± 4.6%, vehicle, 25.63 ± 4.1%, $P = 0.78$), indicating comparable infarct size at baseline. One month following cell injection, FS was significantly enhanced in animals treated with Cymerus MSCs (30.69 ± 1.13% versus 26.08 ± 1.32% in vehicle, $P = 0.01$) but not in those that received BM-MSCs (25.19 ± 1.26% versus 26.08 ± 1.32% in vehicle, $P = 0.63$) (Figure 3B). Moreover, LV dimensions revealed a significant reduction in LV end-systolic diameter (Cymerus MSCs, 0.53 ± 0.01 cm, BM-MSCs, 0.58 ± 0.01 cm, vehicle, 0.56 ± 0.01 cm, $P = 0.05$) but not LV end-diastolic diameter (Cymerus MSCs, 0.77 ± 0.01 cm, BM-MSCs, 0.78 ± 0.01 cm, vehicle, 0.76 ± 0.01 cm, $P = 0.205$] (Figure 3C,D). These findings suggested that intra-myocardial injection of Cymerus MSCs post-MI improved cardiac contractility over a short time frame.

MSCs have no effect on scar size after MI

Large animal and early clinical data in subjects with ischemic cardiomyopathy support the ability of MSCs to impact cardiac function and induce reverse remodeling [15,26,27]. Therefore, to determine whether scar size had an effect on functional improvement with Cymerus MSCs, collagen content was quantified by Gömöri trichrome staining. Unexpectedly, the extent of histological fibrosis did not differ between the Cymerus MSC, BM-MSC and vehicle groups (Figure 4A,B), suggesting an alternate mechanism for the functional improvement.

Transplanted Cymerus MSCs are not arrhythmogenic in the infarcted heart

The risk of cardiac arrhythmias poses a potentially life-threatening problem following cell transplantation [28,29]. Hence, the authors tested cardiac electrical vulnerability *in vivo* by performing burst and extrastimulus ventricular pacing protocols. As shown in Figure 5A, PES was conducted 4 weeks after cell transplantation to assess for potential inducible tachyarrhythmias. There was no
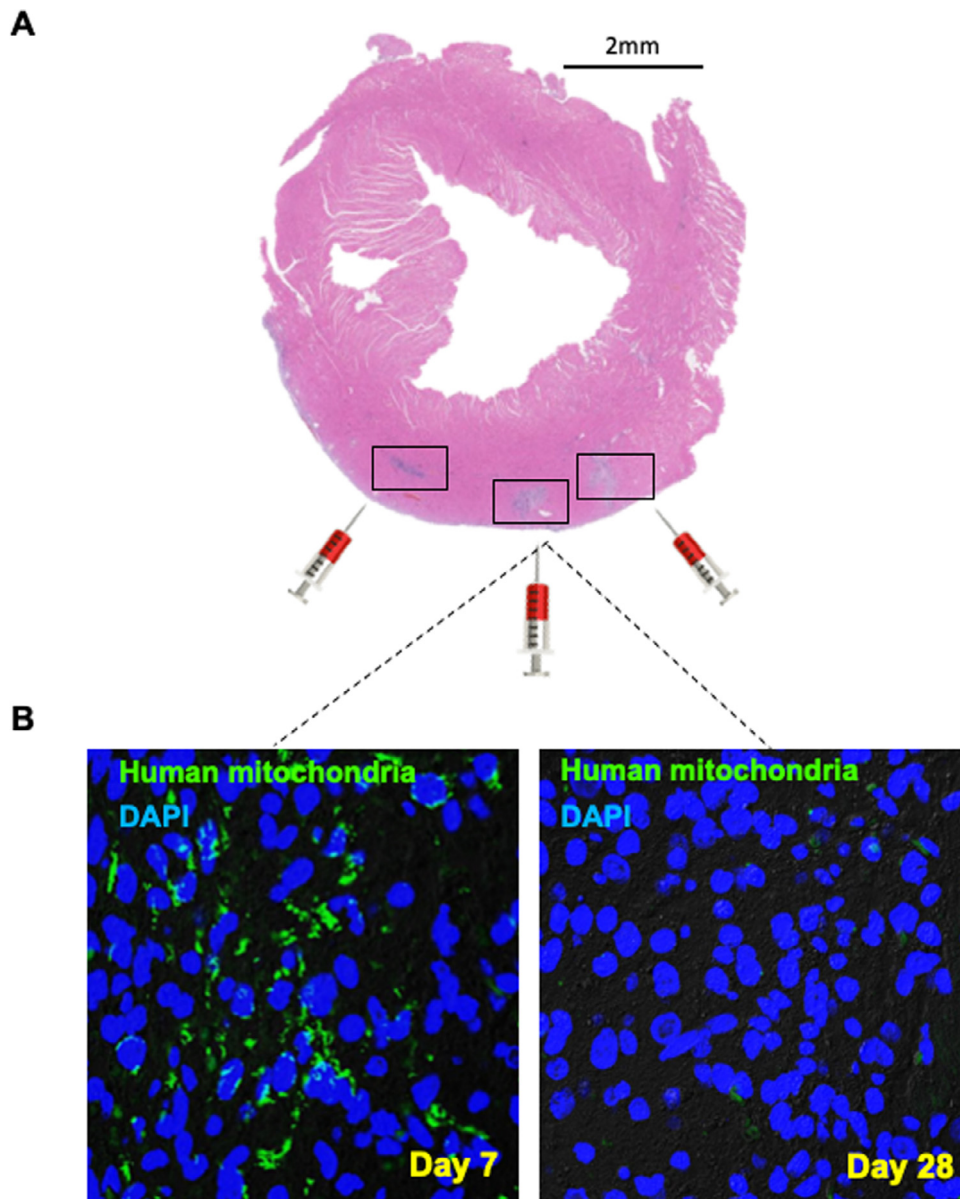
Figure 2. Cymerus MSC pilot engraftment studies. (A) LV cross section stained with H-E, with approximate areas of Cymerus MSC injection at three sites in the anterior wall of the non-infarcted heart shown. (B) Immunofluorescence images showing human mitochondria (green) detected at 7 days but not 28 days after cell transplantation. H-E, hematoxylin and eosin. (Color version of figure is available online).

difference in the percentage of VT between the groups (Cymerus MSCs, 14.2 $\pm$ 0.3%, BM-MSCs, 12.6 $\pm$ 0.2%, vehicle, 13.1 $\pm$ 0.2%, P = 0.94) (Figure 5B). To determine whether there was a difference in distribution of fibrosis (architectural organization of myofibers interspersed with collagen), "heterogeneity index" [25,30] was assessed. There was no difference in the heterogeneity of conducting collagen myofibers (Cymerus MSCs, 0.47 $\pm$ 0.07 cells/mm^2, BM-MSCs, 0.45 $\pm$ 0.05 cells/mm^2, vehicle, 0.46 $\pm$ 0.06 cells/mm^2, P = 0.61) (Figure 5C), suggesting that Cymerus MSCs did not provide an anatomic substrate for re-entrant ventricular arrhythmias. This finding provided an important signal for the safety of this potential cardiac therapy.

Local injection of Cymerus MSCs promotes vasculogenesis in the post-MI heart

Because of the known angiogenic potential of MSCs and lack of mechanistic insight into the functional improvement with Cymerus MSCs, the authors sought to determine the effects of cell transplantation on the post-MI vasculature. The authors measured capillary and arteriolar density in the peri-infarct and remote zones (representative images of peri-infarct zone shown in Figure 6A). As expected, the authors observed a higher number of capillaries (vWF+) in both the peri-infarct Cymerus MSC and BM-MSC-treated groups (Cymerus MSCs, 296 $\pm$ 41 cells/mm^2 versus vehicle, 140 $\pm$ 44 cells/mm^2, P = 0.001 and BM-MSCs, 290 $\pm$ 44 cells/mm^2 versus vehicle, 140 $\pm$ 44 cells/mm^2, P = 0.003) (Figure 6B). Interestingly, however, immunostaining with α-SMA showed that Cymerus MSCs (but not BM-MSCs) enhanced arteriogenesis in the peri-infarct zone (Cymerus MSCs, 16 $\pm$ 2 cells/mm^2 versus vehicle, 6 $\pm$ 2 cells/mm^2, P < 0.0001 and BM-MSCs, 10 $\pm$ 2 cells/mm^2 versus vehicle, 6 $\pm$ 2 cells/mm^2, P = 0.09 and Cymerus MSCs, 16 $\pm$ 2 cells/mm^2 versus BM-MSCs, 10 $\pm$ 2 cells/mm^2, P = 0.01) (Figure 6C). Moreover, the presence of small diameter (6–10 μm) arterioles (Figure 6D) in the Cymerus MSC group suggested arteriolar branching and sprouting of microvessels.
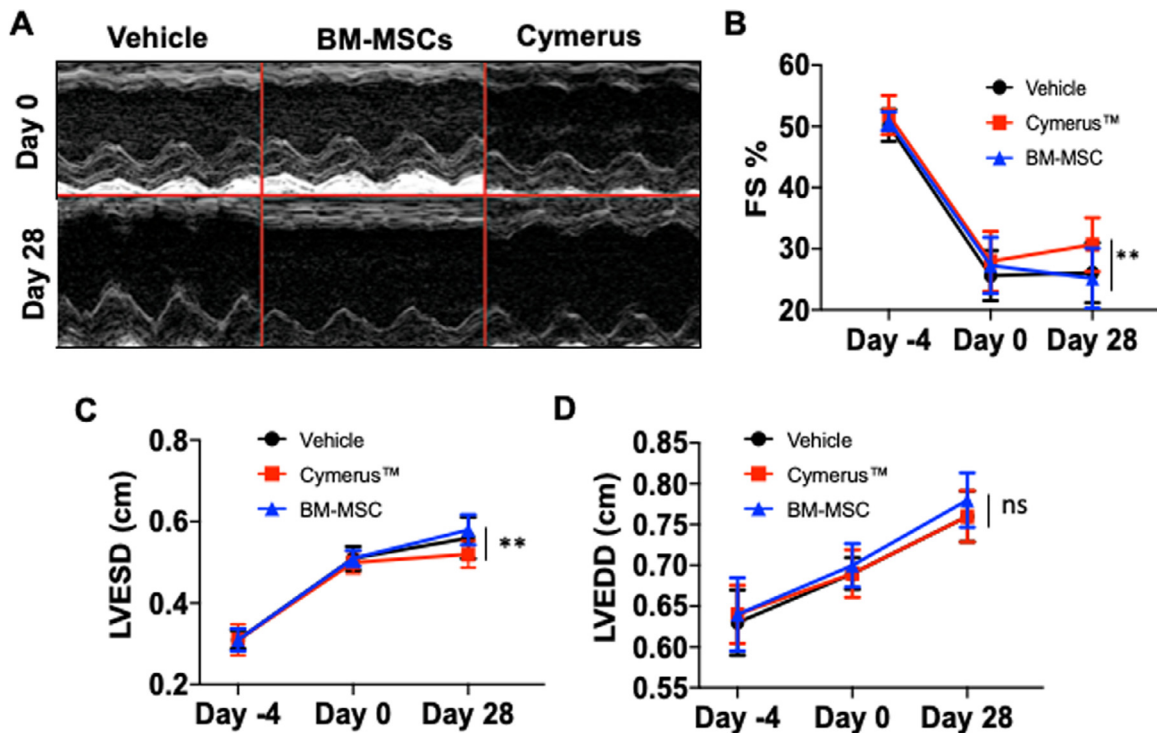
Figure 3. Cymerus MSCs improve LV function after MI. (A) Representative M-mode echocardiographic images showing LV wall motion. (B−D) FS, LVESD and LVEDD are similar between the groups at 4 days prior and day of cell transplantation (day −4 and day 0). Compared with the BM-MSC and vehicle groups, FS significantly improved in Cymerus MSC-treated animals, whereas LVESD decreased at day 28 after cell transplantation. All data are presented as mean ± SEM. A two-tailed, unpaired Student's *t*-test was used to compare between any two groups. One-way ANOVA followed by Holm−Šídák post-hoc test was used to adjust for multiple comparisons. **$P < 0.05$, $^{ns}P = 0.205$. ANOVA, analysis of variance; LVEDD, LV end-diastolic diameter; LVESD, LV end-systolic diameter; SEM, standard error of the mean. (Color version of figure is available online).
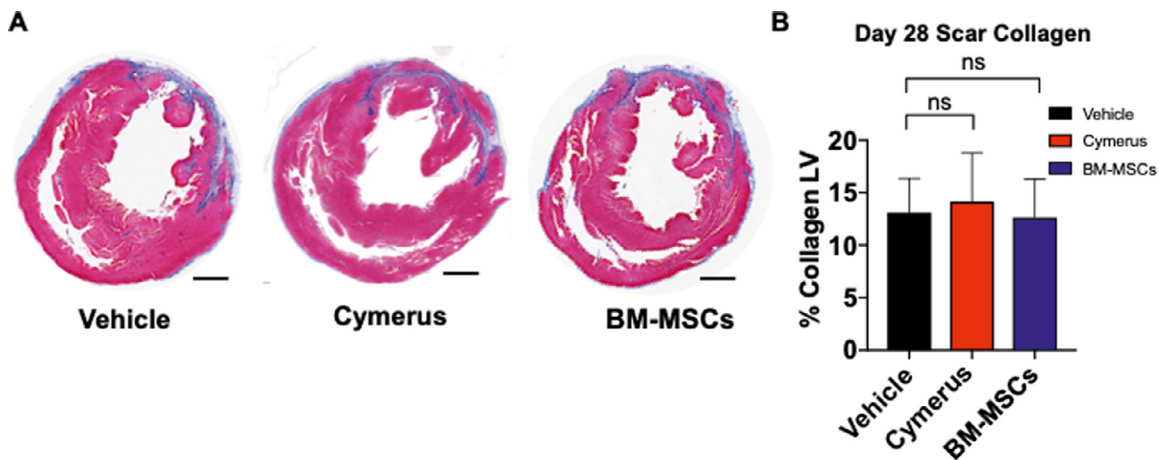

Figure 4. MSCs do not affect scar size after MI. (A) Representative Gömöri trichrome-stained myocardial section of animals 4 weeks after MI. Blue, fibrotic scar tissue; red, viable myocardium. Scale bar = 5 mm. (B) Scar quantification showed no difference in scar size between the groups at day 28 (n = 15 per group in Cymerus MSCs and BM-MSCs, n = 14 in vehicle). All data are presented as mean ± SEM. A two-tailed, unpaired Student's *t*-test was used to compare between any two groups. One-way ANOVA followed by Holm−Šídák post-hoc test was used to adjust for multiple comparisons. $^{ns}P = 0.61$. ANOVA, analysis of variance; SEM, standard error of the mean. (Color version of figure is available online).

Differential cytokine expression profile may explain disparities in angiogenesis and cardiac function between groups

The beneficial effects of MSC transplantation are attributable to the capacity of MSCs to secrete a wide range of cytokines, chemokines and growth factors [31]. To better understand the differential angiogenic effects of Cymerus MSCs compared with BM-MSCs in the post-MI microenvironment, the authors performed protein arrays, probing the secretory profile of each cell type. The expression of 20 different cytokines, chemokines and growth factors in Cymerus MSC- and BM-MSC-conditioned media was compared. Both cell types were

cultured for 2 days in standard culture media to minimize the influence of the media on protein profile. Figure 7A represents a heat map of protein expression normalized to positive control of the assay (biotin-conjugated IgG). Both cell types showed similar cytokine profile expression, which resulted from appropriate derivation of Cymerus MSCs from iPSCs. However, the degree of expression differed from 2- to 4-fold for a number of cytokines, which may be responsible for the enhanced neovascularization. Cymerus MSCs demonstrated expression of IL-8; tissue inhibitor of metalloproteinases (TIMP) 1 and TIMP-2; growth-related oncogene (GRO); regulated upon activation, normal T cell expressed and presumably secreted (RANTES); and
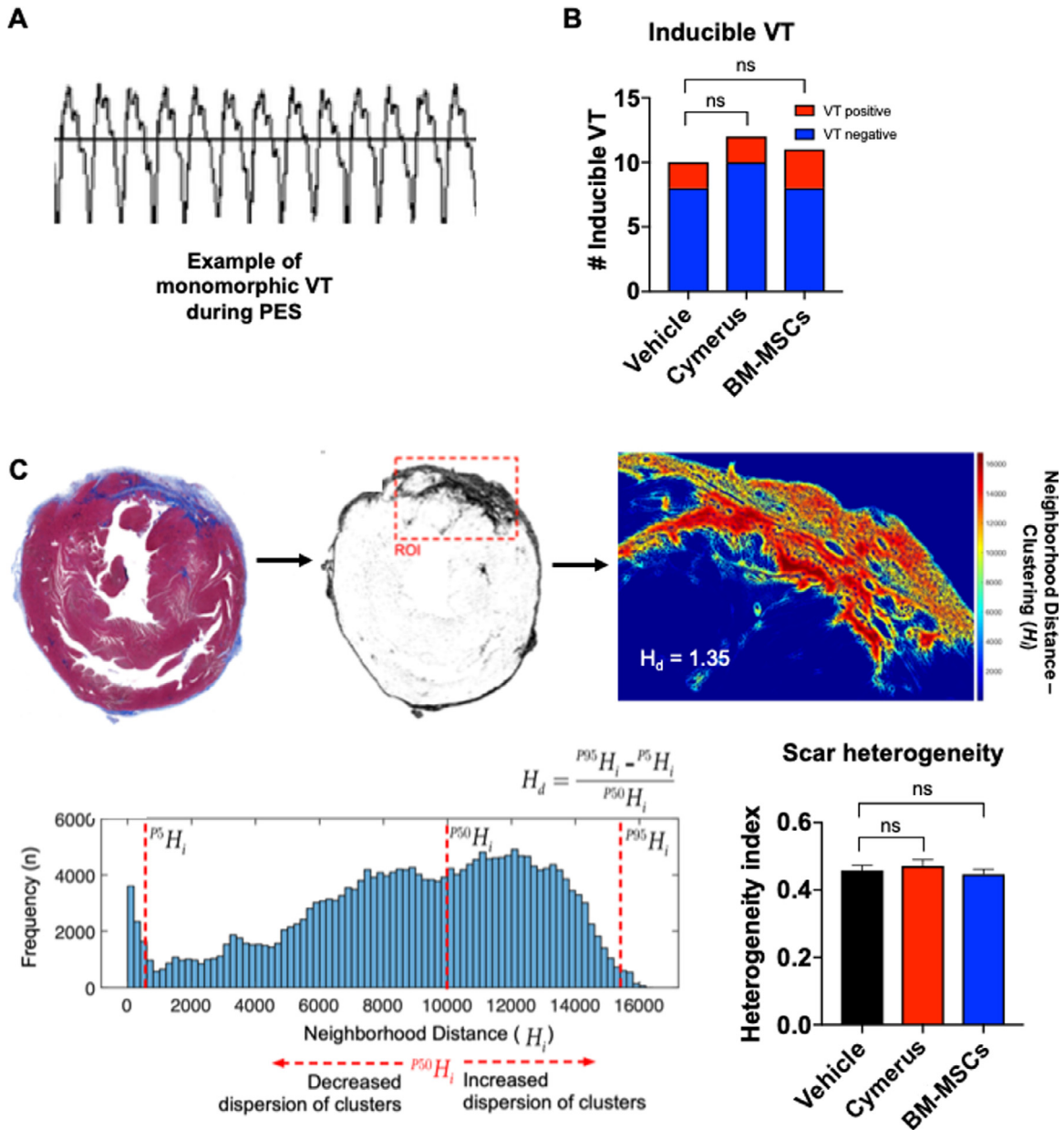
Figure 5. Cymerus MSCs do not provide an anatomic substrate for arrhythmias. (A) Example of monomorphic VT induced by PES in infarcted rats. (B) Quantification of inducible VT (n = 11 in Cymerus MSCs, n = 12 in BM-MSCs, n = 10 in vehicle). (C) Assessment of myocardial scar tissue heterogeneity. Histological cross section of myocardium from the vehicle group indicating myocardial fibrosis (blue) of the lateral LV wall based on Gömöri trichrome tissue differentiation (top left), automated threshold-based detection of scar tissue (top middle) and scar tissue cluster intensity map based on neighborhood distance of collagen (H_i) (top right). Histogram of scar tissue clustering (H_i) and illustrated description of scar heterogeneity (H_d) quantitation (bottom left). Quantification of heterogeneity index (bottom right). All data are presented as mean ± SEM. One-way ANOVA followed by Holm−Šídák post-hoc test was used to adjust for multiple comparisons. [ns]P = 0.94. ANOVA, analysis of variance; ROI, region of interest; SEM, standard error of the mean. (Color version of figure is available online).

vascular endothelial growth factor (VEGF) D to a higher degree, whereas BM-MSCs showed increased expression of IL-6, IL-8 and TIMP-1 and TIMP-2.

When comparing the fold change expression of each protein between the two cell types, upregulation of epidermal growth factor, IL-8, GRO, epithelial neutrophil-activating peptide (ENA) 78, basic fibroblast growth factor (b-FGF), leptin and RANTES and downregulation of VEGF, IL-6, thrombopoietin, TIMP-1 and TIMP-2 were seen in the Cymerus MSC group compared with the BM-MSC group. In particular, Cymerus MSCs showed a 4-fold upregulation of GRO and an approximately 2-fold upregulation of IL-8 and b-FGF (Figure 7B). Downregulation of angiogenesis inhibitors (TIMP-1 and TIMP-2) was also seen in Cymerus MSCs. Cytokines found to be upregulated and

downregulated in Cymerus MSCs (compared with BM-MSCs) are listed in Table 1, with a description of their angiogenic role.

Discussion

Human MSCs have emerged as a promising cell type in regenerative medicine. However, they have several potential shortcomings, including a finite proliferative capacity, necessitating a continual supply of healthy donors [41,42]. The breakthrough of iPSC technology now enables adult somatic cells to be reprogrammed into bona fide PSCs [43,44]. By harnessing iPSCs, it is now possible to generate potentially limitless MSC-like cells from a single human donor [43]. The authors performed this study to test the feasibility of intra-
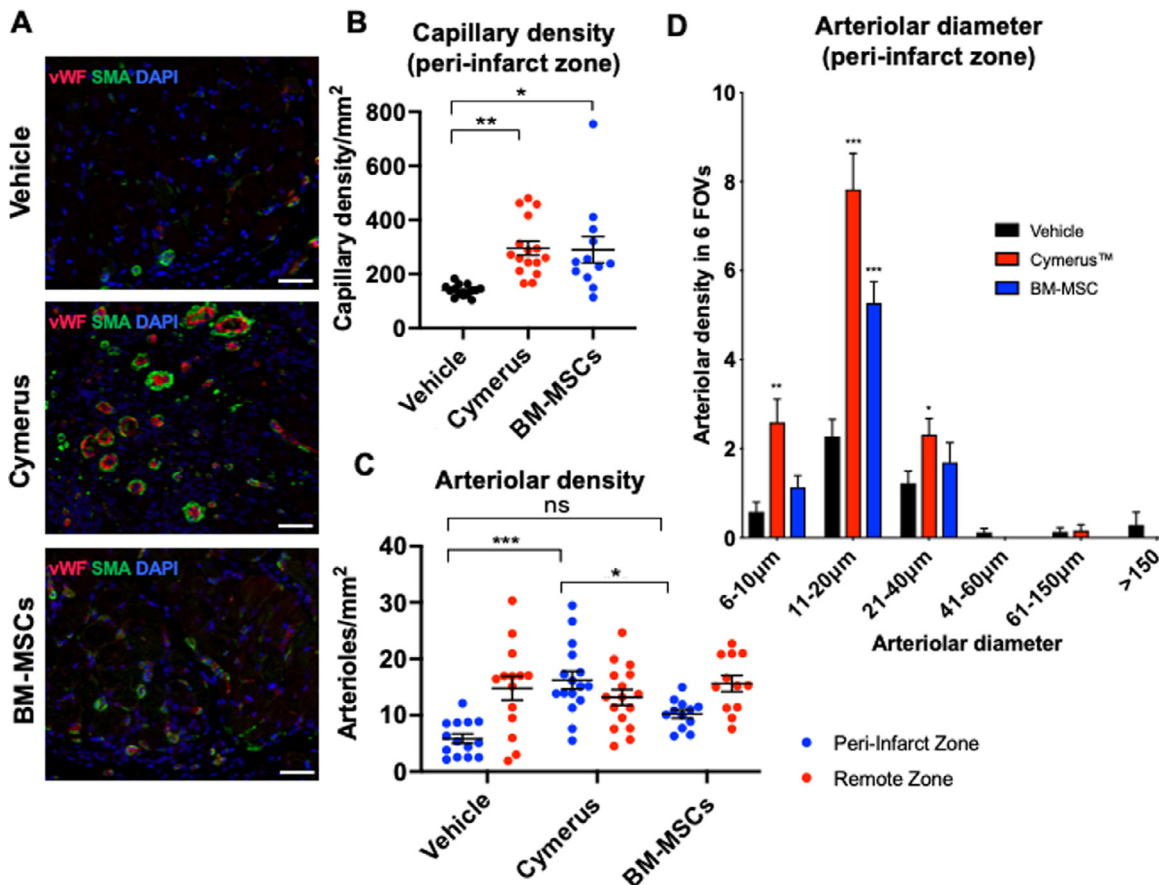
Figure 6. Cymerus MSCs promote vasculogenesis in the post-MI heart. (A) Representative fluorescence images with the presence of vWF+ (red), SMA+ (green) and DAPI (blue) cells in the peri-infarct zone (scale bar = 50 μm), showing that the (B) Cymerus MSC and BM-MSC groups enhance capillary density compared with vehicle at day 28 following cell transplantation (n = 14 per group). (B,C) Long and short horizontal black lines indicate means and SEMs, respectively. (C,D) Quantitative analyses of α-SMA+ vasculature show increased arteriolar density (small- and medium-sized vessels) in Cymerus MSC-treated animals compared with vehicle and BM-MSC groups (n = 14 per group). All data are presented as mean $\pm$ SEM. One-way ANOVA was used to test for multiple comparisons. $^*P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$, $^{ns}P = 0.09$. ANOVA, analysis of variance; DAPI, 4′,6-diamidino-2-phenylindole; FOVs, fields of view; SEM, standard error of the mean. (Color version of figure is available online).

myocardial cell transplantation of iPSC-derived MSCs in a rodent model of MI. In this work, the authors successfully demonstrated that Cymerus MSCs (compared with BM-MSCs) (i) significantly improve LV function despite lack of long-term engraftment, (ii) promote vasculogenesis via upregulation of pro-angiogenic factors and (iii) exhibit a safe cardiac electrophysiological profile.

The retention of regenerative cells and, moreover, engraftment potential have been shown to be fundamental to producing a meaningful therapeutic effect [45]. However, pre-clinical and clinical observations suggest that MSC transplantation can result in robust beneficial effects, including improved contractile function, stimulation of angiogenesis and decreased fibrosis despite rather low engraftment rates [45,46]. In a previous acute MI model in pigs, retention of mesenchymal-like stem cells was higher than that of BM-derived mononuclear stem cells [47], yet within 1 h after intra-myocardial injection the majority of administered cells had left the heart. Hence, the authors' finding of lack of BM-MSC engraftment at 1 month was not surprising. Cymerus MSCs represent a promising cell type not yet tested for cardiac therapeutic indications, and therefore cell engraftment potential was unknown. The authors' results demonstrate that Cymerus MSCs, like BM-MSCs, do not display prolonged persistence following intra-myocardial administration after MI.

Despite the absence of long-term engraftment in both BM-MSC and Cymerus MSC groups, cardiac functional change differed between the groups. Interestingly, cardiac function was similar in the BM-MSC- and vehicle-treated groups at 1 month. By contrast, Cymerus MSC treatment of the infarcted rat heart in the authors' study

resulted in a significant improvement in cardiac function at 1 month despite no reduction in scar size. Meta-analyses using BM-derived progenitor and stem cells (not focused exclusively on MSCs) have demonstrated inconsistent results in the clinic [48]. The absence of standard protocols and differences in timing of cell delivery, delivery method, follow-up and cell processing could explain the variability in performance between MSCs derived from different donors. The heterogeneity of the trials highlights the lack of standardization and demonstrates the great need for large-scale trials showing efficacy prior to moving this field into mainstream medical practice.

There is growing evidence that the beneficial effects of MSC transplantation in the infarcted heart are related to paracrine impact on endogenous cells, resulting in increased angiogenesis and enhanced cell survival [49,50]. The stimulation of angiogenesis is accepted as essential for tissue repair [51]. New vessel formation is initiated by the activation of quiescent vessels in response to angiogenic signals instigated by cytokines and chemokines. Following MI, the lack of oxygen and nutrients precipitates an inflammatory response to enable cardiac repair. Innate immune system cells, including neutrophils and monocytes, release angiogenic factors such as VEGF, IL-8, tumor necrosis factor alpha, hepatocyte growth factor and matrix metalloproteinases [52], which play an important role in new vessel formation. In this respect, MSCs contribute to this phenomenon by releasing paracrine angiogenic signals to recruit monocytes such as RANTES (also known as CCL5), resulting in the release of factors supporting the proliferation of endothelial cells and promoting vasculogenesis [33]. The authors' results show an upregulation of RANTES
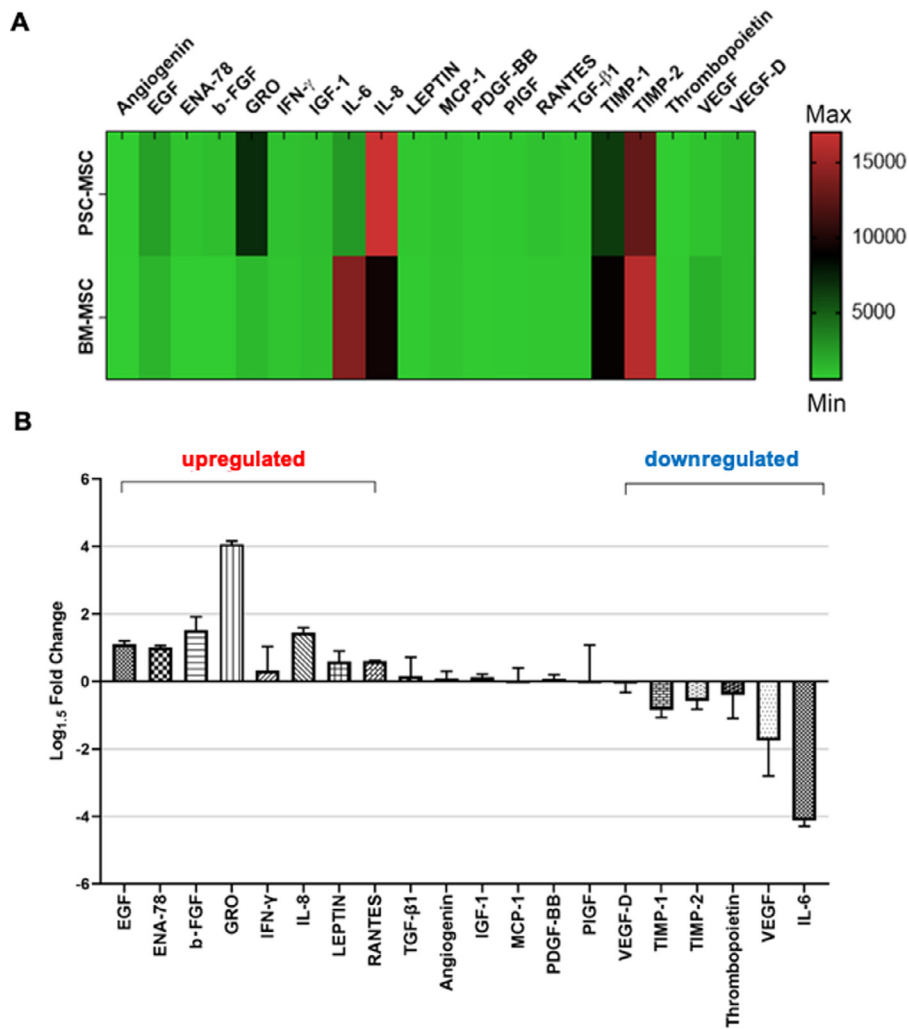
Figure 7. Differential angiogenic cytokine profiles between Cymerus MSCs and BM-MSCs.

(A) Heat map of cytokine expression in conditioned media of Cymerus MSCs and BM-MSCs shown as signal intensities normalized to positive control (biotin-conjugated IgG) signal intensity. (B) Fold change (Log1.5) of upregulated (left) and downregulated (right) cytokines expressed in Cymerus MSCs compared with BM-MSCs. EGF, epidermal growth factor; ENA, epithelial neutrophil-activating peptide; b-FGF, basic fibroblast growth factor; GRO, growth-related oncogene; IFN-γ, interferon gamma; IGF-1, insulin-like growth factor 1; IL, interleukin; MCP-1, monocyte chemoattractant protein 1; PDGF-BB, platelet-derived growth factor BB; PlGF, placental growth factor; TGF-β1, transforming growth factor beta 1; TIMP, tissue inhibitor of metalloproteinases; VEGF, vascular endothelial growth factor; Max, maximum; Min, minimum. (Color version of figure is available online).

Table 1
List of cytokines expressed differently in Cymerus MSCs compared with BM-MSCs.

Cytokine	Angiogenic role
GRO	Recruits angioblasts together with IL-8 in MI areas [32]
IL-8	Pro-angiogenic [32]
RANTES (CCL5)	Recruits monocytes for new vessel formation [33]
Leptin	Promotes vascular tubule formation [34]
b-FGF	Induces vascular regeneration by recruiting and inducing proliferation of ECs [35]
ENA-78 (CXCL5)	Promotes angiogenesis by recruiting neutrophils to the area
EGF	Anti-apoptotic; enhances MSC proliferation and survival [34]
IFN-y	Promotes angiogenesis
IL-6	Promotes endogenous repair by CFs and macrophages to increase generation of new cardiomyocytes and progenitor cells and to rescue apoptotic cells after MI [36]
VEGF	Angiogenesis stimulator that acts by recruiting ECs in the initial stage of the angiogenic response; activity is connected to IL-6 release [37]
Thrombopoietin	Promotes angiogenesis by stimulating VEGF expression in hypoxia conditions [38,39]
TIMP-1 and TIMP-2	Negative regulators of angiogenesis [40]

CFs, cardiac fibroblasts; ECs, endothelial cells; EGF, epidermal growth factor; IFN-y, interferon gamma.

and other pro-angiogenic factors, such as b-FGF, epidermal growth factor, interferon gamma and IL-8, in Cymerus MSCs compared with BM-MSCs. Given a degree of MSC engraftment retention of up to 1 week post-infarction—and within a potential critical phase of LV remodeling—these data provide a rationale for the increased angiogenesis after transplantation of Cymerus MSCs into the infarcted heart.

Interestingly, IL-8 and GRO, which showed the highest fold change between the two cell types, appear to be chemoattractants of angioblasts following MI. Cardiac endothelial cells increase production of the IL-8/GRO-alpha CXC chemokine family, providing a chemoattractant gradient for angioblasts, which are precursors of Cymerus MSCs [32]. In fact, Cymerus MSCs are generated from iPSCs and chemically induced to differentiate into spherical mesenchymal angioblast colonies, which are then harvested and used as precursor cells of both MSCs and endothelial cells. The upregulation of ENA-78 seen with Cymerus MSCs may arise from the fact that iPSCs are capable of indefinite proliferation. These iPSCs in turn provide a potentially infinite source of mesenchymal angioblasts that can then expand into extremely large quantities of MSCs, whereas other types of MSCs have not shown this capability. ENA-78 encodes for the protein CXCL5, which is known to increase vasculogenesis [53]. The

upregulation of ENA-78 seen with Cymerus MSCs may arise from their identity as a more upstream progenitor of vascular and stromal cells compared with their counterpart MSCs derived from adult tissue sources. This may account for the increased vasculogenic potential of Cymerus MSCs compared with BM-MSCs seen in the authors' study. This is of paramount importance from a clinical translational perspective.

With regard to the general cytokine profile, both Cymerus MSCs and BM-MSCs showed high expression of TIMP-1 and TIMP-2, which have previously been reported to be vasculogenesis inhibitors. This may be explained by culture of cells on stiff TCPs. It has been previously reported that adherent cells in an *in vitro* culture environment express metalloproteinases and TIMPs, such as TIMP-1 and TIMP-2, because of the mechanophysical stimuli received from the substrate they are cultured on [40]. TIMP-2 has also been associated with *in vitro* inhibition of endothelial cell proliferation through a mechanism independent of its role in metalloproteinase inhibition [54]. The high stiffness of TCPs could be more similar to injured tissue, such as infarcted heart tissue, as opposed to the stem cell niche in which MSCs usually reside. In the authors' study, both TIMP-1 and TIMP-2 were downregulated in Cymerus MSCs compared with BM-MSCs. Although these *in vitro* experiments cannot recapitulate the *in vivo* environment, these data suggest that the better angiogenesis seen after transplantation of Cymerus MSCs may be TIMP-mediated. VEGF was the first vascular-specific growth factor to be characterized and is widely accepted as the essential driver of vasculogenesis [55]. Similarly, IL-6 is known to stimulate angiogenesis [56,57]. Interestingly, VEGF and IL-6 were downregulated in Cymerus MSCs compared with BM-MSCs (Figure 7B), suggesting that the increased angiogenesis observed with Cymerus MSCs after *in vivo* transplantation is not mediated by VEGF or IL-6.

Although cell-based therapies for cardiac repair have progressed from proof-of-concept to randomized clinical trials, arrhythmogenesis has always been a feared complication [58]. Limited pre-clinical work demonstrated varied arrhythmic effects that may be explained by the types of stem cells (skeletal myoblasts, BM-derived cells) and different routes of administration used [59,60]. Early clinical studies with skeletal myoblast implants then set a precedent that endocardial stem cell injections may provoke arrhythmia due to heterogeneous conduction and repolarization, which could serve as a substrate for arrhythmia [61]. The authors' study showed that cardiac transplantation of Cymerus MSCs (and BM-MSCs) did not increase cardiac arrhythmogenicity compared with the vehicle-treated group. This is in keeping with data sets from the POSEIDON and TAC-HFT randomized clinical trials demonstrating no pro-arrhythmic effects from MSC transplant occurring in either the short term (within days) or long term (up to 1 year) [45,58,62]. Moreover, the lack of pro-arrhythmia seen with Cymerus MSCs is a promising result despite the higher resting heart rate in rodents [63], which could potentially affect the electromechanical function of implanted cells. In contrast to MSCs, PSC-derived cardiomyocytes have had significant barriers to translation, as evidenced by graft-related tachyarrhythmias despite significant remuscularization [28,29].

The limitations of this study are important to consider. Firstly, experiments to investigate any possible effects caused by different vehicles used for the different cell groups (Plasma-Lyte used for Cymerus MSCs and DMEM medium for BM-MSCs) was not performed. Further *in vivo* and *in vitro* experiments are required to assess the effects of Plasma-Lye and/or DMEM on functional outcome, vasculogenesis and cytokine profile. Second, although the improvement in LV function seen with Cymerus MSCs may be related to a pro-arteriogeneic response, further mechanistic studies are required to determine whether other factors, such as immunomodulatory effects and scar alignment, may play a role in the therapeutic effect [64−69]. Long-term studies in a large animal model and/or human clinical trial are required to definitely test cell retention strategies,

confirm functional improvements and assess safety. Nevertheless, these proof-of-concept studies demonstrate feasibility and pave the way for future translational studies designed for regulatory approval of this therapy.

Conclusions

The authors have demonstrated that intra-myocardial administration of iPSC-MSCs (Cymerus MSCs) provides a better therapeutic effect compared with conventional BM-MSCs in a rodent model of MI. In the absence of engraftment, the therapeutic effects of Cymerus MSCs *in vivo* appear to be related to their ability to improve neovascularization via paracrine mechanisms. *In vitro* studies using serum-free conditioned medium of Cymerus MSCs show a pro-angiogenic secretory profile with an upregulation of pro-angiogenic factors and downregulation of metalloproteinases (TIMP-1 and TIMP-2). Given manufacturing and scalability advantages, iPSC-MSC therapy offers an exciting opportunity for "off-the-shelf" stem cell therapy, rendering it an attractive cell type for cardiac repair.

Author Contributions

Conception and design of the study: JJHC. Acquisition of data: ST, TTYL, PB, AMB, PF, FNR, JP and SR. Analysis and interpretation of data: ST, TTYL, PB, AMB, PF, SR, JJHC, KK and EK. Drafting or revising the manuscript: ST, JJHC, TYLL, SR, KK and EK. All authors have approved the final article.

Data availability

All data associated with this study are present in the article or supplementary materials.

Declaration of Competing Interest

The authors have no commercial, proprietary or financial interest in the products or companies described in this article.

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