Dual stem cell therapy synergistically improves cardiac function and vascular regeneration following myocardial infarction

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Since both myocardium and vasculature in the heart are excessively damaged following myocardial infarction (MI), therapeutic strategies for treating MI hearts should concurrently target both so as to achieve true cardiac repair. Here we demonstrate a concomitant method that exploits the advantages of cardiomyocytes derived from human induced pluripotent stem cells (hiPSC-CMs) and human mesenchymal stem cell-loaded patch (hMSC-PA) to amplify cardiac repair in a rat MI model. Epicardially implanted hMSC-PA provide a complementary microenvironment which enhances vascular regeneration through prolonged secretion of paracrine factors, but more importantly it significantly improves the retention and engraftment of intramyocardially injected hiPSC-CMs which ultimately restore the cardiac function. Notably, the majority of injected hiPSC-CMs display adult CMs like morphology suggesting that the secretomic milieu of hMSC-PA constitutes pleiotropic effects in vivo. We provide compelling evidence that this dual approach can be a promising means to enhance cardiac repair on MI hearts.
Myocardial infarction (MI) is a fatal disorder that inflicts a permanent loss of cardiomyocytes (CMs) and scar tissue formation, resulting in irreversible damage to cardiac function ensued by heart failure. While cardiac regeneration is considered unfeasible with current medical options, accumulating evidence in animal models as well as clinical trials continue to demonstrate that stem cells could offer new opportunities for treating MI hearts.

Among them, human mesenchymal stem cells (hMSCs) have long been considered a promising candidate for cell-based therapy aimed to their beneficial paracrine factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF2), and hepatocyte growth factor (HGF) that promote angiogenesis, neovascularization, and cell survival. hMSCs are also known to secrete potent anti-fibrotic factors including matrix metalloproteinases 2, 9, and 14, which inhibit the proliferation of cardiac fibroblasts thereby attenuating fibrosis.

In tandem, CMs derived from human pluripotent stem cells (hPSC-CMs), which include both human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSC), are propitious due to their similarities with human primary CMs as to expressions of cardiac-specific genes, structural proteins, and ion channels as well as spontaneous contraction. Several preclinical studies have shown that hPSC-CMs successfully engraft, align, and couple with host myocardium in a synchronized manner to improve cardiac function.

Since the heart is an organ composed of cardiac muscles and blood vessels, both cardiac muscles and vasculatures in the heart were excessively damaged following MI. Thus therapeutic strategies for treating MI hearts should be focused to comprehensively repair all that together for achieving true cardiac repair. The principle behind cell-based cardiac regeneration therapy should adhere to the same principles as well.

Hence, in this study, we develop a multipronged approach aiming to concurrently rejuvenate both the myocardium and vasculatures utilizing both hiPSC-CMs and hMSCs. We hypothesize that while intramyocardially injected hiPSC-CMs would restore heart function by engraftment with the host myocardium, epicardially implanted hMSC patches (hMSC-PA) would simultaneously enhance vascular regeneration through consistent secretion of angiogenic paracrine factors in MI-induced hearts. We demonstrate that the dual approach of applying hiPSC-CMs as well as hMSC-PA lead to a significant improvement of cardiac function and enhancement of vessel formation post MI. Of note, not only do the implanted hMSC-PA significantly increase the retention of intramyocardially injected hiPSC-CMs but also preserve injured host CMs via paracrine release of cytokines and growth factors, resulting in functional improvement. Surprisingly, the majority of injected hPSC-CMs neighboring hMSC-PA display the rectangular-shaped morphology of adult-like CM and significant expression of Gja1, a major gap junction protein, suggesting that hMSC-PA promotes CM maturation in vivo. Furthermore, we identify that paracrine factors released from hMSC-PA constitute the key contributors with pleiotropic effects, including pro-angiogenesis, anti-inflammation, anti-fibrosis, and CM maturation. These results carry significant implications for stem cell therapy in cardiac repair, highlighting the need for intricate and strategic designs to achieve complete restoration.

Results
Selection of optimal hMSCs and their characterization. To select the best candidate of hMSCs in terms of secretion of paracrine factors, we examined distinct types of hMSCs isolated from different sources such as human turbinate, human adipose tissue, and human bone marrow and compared the concentration of VEGF secreted to their conditioned medium. As a result, we found that concentration of VEGF released from hMSCs derived from human bone marrows were substantially higher than other types of hMSCs (Supplementary Fig. 1A). Subsequently, we characterized hMSCs from bone marrows and found that bone marrow-derived hMSCs exhibited evident phenotypes of hMSCs. Cultured bone marrow-derived hMSCs displayed a homogeneous spindle-like shape, which is a typical cell morphology of hMSCs and express abundant expression of several specific markers for hMSCs, such as CD73, CD105, CD90, and CD44 (Supplementary Fig. 1B).

hMSC-PA secrets paracrine factor. First, to examine the viability of hMSCs within the hMSC-PA, we generated hMSC-PA (Supplementary Fig. 2A) and performed the live/dead staining. As a result, we verified that the majority of hMSCs within the hMSC-PA remained alive and less than one-tenth of cells were dead, indicating that hMSC-PA can maintain the viability of hMSCs. Next, to confirm whether hMSC-PA could efficiently release paracrine factors from embedded hMSCs, we cultured the hMSC-PA in vitro, collected supernatants, and performed VEGF ELISA assay (Supplementary Fig. 2B). As a result, we verified that hMSC-PA released VEGF over time. We observed that the concentration of VEGF released from hMSC-PA increased in a time-dependent manner, suggesting that hMSC-PA is capable of releasing sufficient amounts of cytokines (Supplementary Fig. 2B).

hMSC-PA increases the expressions of multiple factors. To explore the paracrine effects of hMSC-PA in MI hearts in vivo, we first performed gene expression analyses with rat heart tissues harvested 7 days after hMSC-PA implantation into MI hearts (Fig. 1a–d). qRT-PCR results show that hMSC-PA significantly upregulated the expression of several angiogenesis-related genes, such as vascular endothelial growth factor A (VEGFa), insulin-like growth factor 1 (IGF-1), fibroblast growth factor 2 (FGF2), placental growth factor (PLGF), angiopoietin 1 (Ang1), and angiopoietin 2 (Ang2), and cluster of differentiation 31 (CD31) in comparison with the MI control hearts, as well as the cell-free patch implanted group (Fig. 1e). In addition, while expression levels of pro-inflammatory-related genes such as interferon gamma (IFNG), interleukin 1 beta (IL-1b), and tumor necrosis factor alpha (TNFa) were not significantly altered, the expression levels of anti-inflammatory-related genes including transforming growth factor beta 1 (TGFβ1) and interleukin 10 (IL10) were significantly increased in hMSC-PA implanted hearts (Fig. 1f). Of interest, hMSC-PA significantly downregulated the expression of several anti-fibrosis-related gene including collagen type 1 (COL1) and collagen type 3 (COL3) and increased the expression of tissue inhibitors of metalloproteinase 2 (TIMP-2) (Fig. 1g).

These qRT-PCR data clearly suggest that hMSCs-PA could induce significant paracrine effects for multiple factors regarding angiogenesis, inflammation, and fibrosis in MI hearts.

hMSC-PA is only effective in improving angiogenesis. Given the instant increased expression of several angiogenesis-related genes, we sought to investigate whether hMSC-PA could improve vascular regeneration in MI hearts. To determine the effects of hMSC-PA, we perfused isoelectric B4 (IsB4) conjugated with green fluorescent dye into the heart tissues to visualize the vessels prior to tissue harvest at 8 weeks. Consistent with the qRT-PCR results, assessment of fluorescent images showed that the number of capillaries in the infarct zones of hMSC-PA implanted hearts were significantly higher than the untreated control group suggesting hMSC-PA have significant effects on vascular regeneration in MI hearts (Fig. 2a). To ensure that the illustrated
Implantation of hMSC-PA into MI hearts enhances the expression of multiple factors. a Preparation of heart-derived decellularized extracellular matrix (hdECM) bioink. b Macroscopic view and illustration of 3D printing system used to produce PCL platform. c Schematic illustration of human mesenchymal stem cell patch (hMSC-PA). Scale bar: 4 cm. d Epicardially implanted hMSC-PA in MI heart at 1 week. e-g Quantitative real-time polymerase chain reaction analysis of relative mRNA expression of multiple factors in the myocardium at 1 week after hMSC-PA implantation in MI induced hearts. e Angiogenesis (f) inflammation, (g) fibrosis. The y-axis represents relative mRNA expression of target genes to GAPDH. A.U. indicates arbitrary units. The data are represented as mean ± SEM. *p < 0.05 compared with MI group, **p < 0.05 compared with PA-only group; n = 3 biologically independent samples per group. One-way ANOVA was used for statistical analyses. Control: MI control, PA-only: cell-free hdECM patch, and hMSC-PA: hMSC-loaded patch

Next, to evaluate cardiac function and cardiac remodeling, we performed echocardiography on a regular basis. While there was a substantial increase of capillary density in hMSC-PA implanted MI hearts, the echocardiography results demonstrated that hMSC-PA did not induce functional improvement in MI hearts. Both ejection fraction (hMSC-PA: 33.93% ± 1.66% vs. 32.21% ± 0.67% in control group, n = 7 animals; p < 0.05; one-way ANOVA was used for statistical analyses) and fractional shortening (hMSC-PA: 14.07% ± 0.81% vs. 11.56% ± 0.94% in control group, n = 7 animals; p < 0.05; one-way ANOVA was used for statistical analyses) were not significantly different with the untreated control group (Fig. 2b). Based on these results, we postulated that although implantation of hMSC-PA is very effective for vascular regeneration, it is insufficient to improve cardiac function and requires additional cell sources to further enhance heart function.

Dual approach increases cardiac function and angiogenesis. Subsequently, we investigated the therapeutic potential of the dual approach by intramyocardially injecting hiPSC-CMs and implanting hMSC-PA (Supplementary Movie 3). After the induction of MI by LAD ligation, we generated five experimental groups receiving: (i) Sham control, (ii) untreated control, (iii) hiPSC-CM intramyocardial injections, (iv) hMSC-PA implantations, or (v) both hiPSC-CMs and hMSC-PA, and compared their therapeutic effects. First, echocardiography results showed that cardiac function in the combined treatment group was significantly higher than hiPSC-CM only or hMSC-PA-only group, as determined by ejection fraction (hiPSC-CMs (CM): 35.95% ± 1.85% vs. combined (CM + PA): 43.15% ± 0.52% vs.

Generation of cardiomyocyte derived from hiPSCs. Owing to several previous studies reporting the promising effects for restoring cardiac function in MI hearts, we sought to test hiPSC-CMs as an additional cell type to treat MI hearts. Accordingly, we generated hiPSC-CMs by using a small-molecule-based 2D differentiation method (Supplementary Fig. 5).9-11 Spontaneous beating was observed around differentiation day 7 (Supplementary Fig. 5B and Supplementary Movies 1, 2). Immunostaining demonstrated that most differentiated hiPSC-CMs displayed CM-specific marker proteins, such as TNNT2 and ACTN2 (Supplementary Fig. 5C). Furthermore, quantification analyses performed via flow-cytometry analysis demonstrated an upwards of 98% of hiPSC-CMs were positive for TNNT2, and ~84% of hiPSC-CMs expressed MYL2, a well-known marker for ventricular CMs suggesting successful generation of highly purified hiPSC-CMs (Supplementary Fig. 5D).
ingly, we found that implantation of hMSC-PA signifi-
cantly enhance the engraftment of hiPSC-CMs.

The number of capillaries was counted per mm² within the infarct zone. Scale bars: 50 μm. The data are represented as mean ± SEM. *p < 0.05 compared with control group; n = 5 biologically independent samples per group. T test was used for statistical analyses. a Rats undergoing MI were implanted with a hMSC-PA or control, followed by echocardiography analysis. Both ejection fraction (EF) and fractional shortening (FS) were not significantly higher than control group. The data are represented as mean ± SEM. *p < 0.05 compared with Sham group; n = 5 animals per group. One-way ANOVA was used for statistical analyses. Sham Sham operation, MI CON MI control, PA hMSC-loaded patch.

Moreover, histological images demonstrated that the number of capillaries (mm²) in the both border zone and infarct zone of the hearts from the complementary group were substantially higher than the control and the hiPSC-CMs only group (Fig. 3b). The number of functional capillaries (diameter range: 5–10 μm) in the complementary group was significantly higher than other test groups (Supplementary Fig. 7). Importantly, the combined treatment group significantly decreased cardiac fibrosis. The results from Masson’s trichrome staining using cardiac tissue harvested at 8 weeks exhibited an area of fibrosis (%) was significantly lower in combined treatment groups compared with other experimental groups (Fig. 3c; Supplementary Fig. 8 and Supplementary Movies 4, 5). Collectively, these results clearly indicate that the combined treatment lead to comprehensive cardiac repair through the improvement of cardiac function, as well as vascular regeneration and reduction of cardiac fibrosis.

**hMSC-PA enhance the engraftment of hiPSC-CMs.** Interestingly, we found that implantation of hMSC-PA significantly improved the retention of intramyocardially injected hiPSC-CMs. The injected hiPSC-CMs were tracked in heart tissues by using two distinct types of hiPSC-CMs: (1) hiPSC-CMs continuously expressing the green fluorescence signal (hiPSC-CMs-GFP) (Supplementary Fig. 9 and Supplementary Movies 6, 2) hiPSC-CMs pre-labeled with a potent red fluorescence dye, CM-DiI, prior to cell injection (Supplementary Fig. 10). Immunostaining with human-specific antibodies for the MYH7 protein and mitochondria further verified the identity of hiPSC-CM-GFP as human CMs (Fig. 4a, b). Next, the number of hiPSC-CMs that remained in heart tissues was quantified at different time points by harvesting the samples obtained from two different experimental groups (CM vs. CM + PA) at 2, 4, and 8 weeks after administration. Cell counting was conducted manually, and there was significant difference in the number of hiPSC-CMs between the two groups. The CM + PA group displayed a substantially higher count than that of the CM group (Fig. 4c). Notably, the CM + PA group also showed hiPSC-CMs distributed throughout all regions of the left ventricle while the CM group showed hiPSC-CMs only localizing near the injection sites (Supplementary Fig. 10). Both groups were able to retain the majority of hiPSC-CMs within the infarct zone and the cells remained viable until 8 weeks following injection as evidenced by the TUNEL assay (Fig. 4d).

**hMSC-PA improves the maturity of hiPSC-CMs.** Remarkably, immunohistochemistry with TNNI2 and MYH6/7 antibodies revealed that the CM + PA group evidently displayed a more mature form (Fig. 4e; Supplementary Fig. 11). While the morphology of hiPSC-CMs in the CM group exhibited a typically immature globular phenotype, the CM + PA group exhibited a much larger rod-shaped structure that resembled adult-like CMs.
These effects were also evident in vitro when 10 or 30% of hMSC-conditioned media (hMSC-CM) collected from hMSC culture greatly increased the size of cultured neonatal rat ventricular cardiomyocytes (NRVM). The expression level of genes related to CM maturation was not altered in any significant way in exception to cardiac troponin T (TNNT2). Collectively, these results indicate the proenectic milieu secreted by hMSCs are a dominant factor in CM maturation (Supplementary Fig. 12).

More importantly, concomitant staining with Gja1, a major gap junction protein, further showed that a substantial number of engrafted hiPSC-CMs in the CM + PA group formed gap junctions with host CMs (Fig. 4f; Supplementary Fig. 13). Limited expression of Gja1 in the CM group suggests the supportive and essential role of hMSC-PA in the formation of gap junctions between the injected hiPSC-CMs and host CMs (Fig. 4f; Supplementary Fig. 13). In addition, the results from multi-electrode arrays (MEAs) using cocultured hiPSC-CMs and NRVM in vitro exhibited a well-synchronized action potential, suggesting a well-coupled syncytium between two CMs. No visible sign of arrhythmogenic beating was detected during the recording period (Supplementary Fig. 14 and Supplementary Movie 7). Taken together, these results suggest that hMSC-PA can markedly enhance cell retention, improve functional maturation, and induce integration with the host myocardium in MI hearts.

Cytokines secreted from hMSCs increases angiogenesis. To identify detailed therapeutic mechanism of hMSC-PA, we performed various types of in vitro analyses. Among the first, to test whether hMSCs directly promote angiogenesis, we conducted cell migration and tube-formation assays using conditioned media (CM) collected from cultured hMSCs (hMSC-CM). At first, we performed the scratch assay, an in vitro experiment for measuring endothelial cell (ECs) proliferation/migration, which are critical steps of angiogenesis. As shown in Fig. 5a, the addition of 10% hMSC-CM significantly enhanced the migration of HUVEC and induced faster closure of the cell-free gap compared with the control group, suggesting that cytokines secreted by hMSCs enhanced the mobility of ECs (Fig. 5a). Next, the results from Matrigel tube-formation assay, which was carried out to evaluate vessel-forming capability, showed that tube length and branches assessed 9 h later were significantly higher in the 10% hMSC-CM-treated HUVEC compared with untreated control HUVEC (Fig. 5b).

The effects of hMSCs for EC proliferation were further examined by our co-culture system with hMSCs and HUVEC. As shown in Fig. 5c, the proliferation rate of HUVEC was examined by our co-culture system with hMSCs and HUVEC. To confirm detailed therapeutic mechanism of hMSC-PA, we performed multi-electrode arrays (MEAs) using co-culture of hMSCs and HUVEC. Next, the results from Matrigel tube-formation assay, which was carried out to evaluate vessel-forming capability, showed that tube length and branches assessed 9 h later were significantly higher in the 10% hMSC-CM-treated HUVEC compared with untreated control HUVEC (Fig. 5b).
roughly the same rate ($\times 10^5$), but a clear difference in growth rate appeared on day 2. The size of HUVEC droplet and cell number in the co-culture plate was markedly higher than those in monoculture of HUVEC, indicating that the factors secreted from hMSCs have significant vasculogenic potential by proliferation and migration of ECs. Collectively, these results indicated that enhanced vessel formation shown in a number of in vitro assays was due to the angiogenic factors secreted from hMSCs, which is consistent with our in vivo results.

**hMSC-secreted factors improve the survival of hiPSC-CMs.**

Given the ability of hMSC-PA to improve the survival and retention of injected hiPSC-CMs, we sought to examine whether hMSC-CMs exerted direct cytoprotective effects in hiPSC-CMs in vitro. Ischemic injury was simulated by exposing hiPSC-CMs to H$_2$O$_2$. Administration of hMSC-CMs significantly improved cell viability as determined by the Annexin V assay and lactate dehydrogenase (LDH) release. As shown in Fig. 6, treatment with 10% of hMSC-CM substantially decreased the number of both PI and Annexin-positive cells (Fig. 6a). Furthermore, the results from LDH assays revealed that LDH released from hMSC-CM-treated hiPSC-CMs was significantly lower than untreated control hiPSC-CMs, suggesting hMSC-CM possess cardioprotective effects against ischemic insults (Fig. 6b). We also observed that cytokines released by hMSCs were able to induce CM migration in a chemotactic manner as hiPSC-CMs began to migrate towards hMSCs after 12 h and reached the center of the dish at 72 h. Within 48 h, hiPSC-CMs (black arrow) continued to approach the periphery of hMSCs which is indicative of cell–cell affinity (Supplementary Fig. 15).

**Cytokine array in BM-MSC conditioned medium.**

Lastly, to identify the panel of factors secreted by hMSCs, we prepared the hMSC-CM from hMSC cultures at two different time points at day 7 and 14 and analyzed them via Proteome Profiler Human Arrays (Fig. 7). The following families of cytokines were observed from hMSC-CM: angiogenesis (angiopeptin 1, vasorin, progranulin, IGFBP-2, IGFBP-7, VEGF, DKK-1, DKK-3, IL-8, uPA), ECM remodeling (MMP-1, MMP-13, MMP-20, thrombospondin-1, TIMP-1, TIMP-2, TIMP-3, latent TGF-beta, bp1), cell viability (EDA-A2, GDF-15, IL-1 sRII, MCP-1, MIP-2), and inflammation (IL-28A, lymphotactin, activin A, GRO) (Fig. 7).

**Discussion**

In this study, we demonstrate a approach in treating MI that exploits the providential advantages of both hiPSC-CMs and...
Fig. 5 Secreted factors from hMSCs promote endothelial cell migration and vasculogenic potential. a Endothelial cell-migration assay. The HUVEC were incubated with 10% conditioned media harvested from hMSC cultures (hMSC-CM) or control media to evaluate the migration capability. Representative Images under an inverted microscope and quantification summary. The data are represented as mean ± SEM. *p < 0.05 compared with untreated control group; n = 5 biologically independent samples per group. T test was used for statistical analyses. b Matrigel plug assay. The 10% hMSC-CM was treated to HUVEC on matrigel to examine the vasculogenic potential. Representative images of tubes formed on Matrigel and quantification summary. The data are represented as mean ± SEM. *p < 0.05 compared with hMSC basal media, #p < 0.05 compared with EGM2 group; n = 3 biologically independent samples per group. One-way ANOVA was used for statistical analyses.

Fig. 6 Direct cytoprotective effects of the hMSC-conditioned medium on hiPSC-CMs undergoing simulated ischemic injury. a, b Treatment with hMSC-conditioned media (hMSC-CM) increased cell survival after H2O2 (200 µM) treatment as determined by the (a) Annexin V and (b) lactate dehydrogenase (LDH) assay. The data are represented as mean ± SEM. *p < 0.05 compared with untreated control group, #p < 0.05 compared with H2O2-only treated control group; n = 3 biologically independent samples per group. One-way ANOVA was used for statistical analyses.

hMSC-PA to significantly amplify cardiac repair. Synergic effects of intramyocardially injected hiPSC-CMs and epicardially implanted hMSC-PA collectively rejuvenated the myocardium and vessels post MI. Epicardially implanted hMSC-PA provided a complimentary microenvironment which enhanced vascular regeneration through prolonged secretion of beneficial paracrine factors as expected, but more importantly it improved the retention, distribution, engraftment, and maturation of hiPSC-CMs which ultimately augmented heart function and restored the injured myocardium (Fig. 8).
population over time to dwindling levels by 8 weeks, whereas its presence greatly bolstered the number of survivals for subsequent engraftment (Fig. 4c). From this, we presumed that the paracrine factors secreted by hMSC-PA improved the survival and engraftment of hiPSC-CMs by enabling the cells to more resistant to the hostile microenvironment in ischemic tissues, particularly during the early stage of implantation, which is critical for cell engraftment and survival. We also envisioned that the placement hMSC-PA over the epicardium may have also served as a biophysical barrier preventing the mechanical expulsion of injected hiPSC-CMs into the epicardial space, further contributing to the increased retention rate of hiPSC-CMs.

Notably, we found that hMSC-PA promoted the maturation of injected hiPSC-CMs in MI hearts. Our histological analyses results demonstrated that hiPSC-CMs together with hMSC-PA led to a more elongated and rectangular cell shape, which are typical morphological characteristics of matured adult CMs. Those hiPSC-CMs stained more strongly for CM-specific markers such as c-MYH6/brils28.

Indeed, there have been several previous studies that describe the beneficial effects of hPSC-CMs5–8 or hMSCs14–16 or other cell types, such as cardiac progenitor cells17,18, endothelial cells19–21, and smooth muscle cells20,21, on MI either separately or as a combinatory20–22. These individual type of cells or cell mixtures were delivered to the hearts through direct intramyocardial injection5–8 or as a patch form19,21,23 formulated by using several different types of biomaterials. Compared with those previous reports, this study is, to the best of our knowledge, the first to simultaneously examine the effects of two distinct major stem cell types delivered via two different routes for inducing comprehensive cardiac repair.

A primary obstacle to cell-based cardiac therapy is the extremely low rate of retention and engraftment to the host myocardium which is particularly important because degree of cell-based cardiac repair largely depends on the number of cells that survive and engraft within the heart24–26. In this regard, our histological results revealed a substantial increase in the retention and thereby engraftment of intramyocardially injected hiPSC-CMs when paired with epicardial hMSC-PA. The absence of hMSC-PA resulted in a rapid decline of the hiPSC-CM population over time to dwindling levels by 8 weeks, whereas its presence greatly bolstered the number of survivals for subsequent engraftment (Fig. 4c). From this, we presumed that the paracrine factors secreted by hMSC-PA improved the survival and engraftment of hiPSC-CMs by enabling the cells to more resistant to the hostile microenvironment in ischemic tissues, particularly during the early stage of implantation, which is critical for cell engraftment and survival. We also envisioned that the placement hMSC-PA over the epicardium may have also served as a biophysical barrier preventing the mechanical expulsion of injected hiPSC-CMs into the epicardial space, further contributing to the increased retention rate of hiPSC-CMs.

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Even though this study evidenced several promising results, there are some limitations to the study that require consideration. Since our complementary approach (CM + PA) was examined in a permanent ischemia model, the outcomes may be different if applied to models with advanced heart failure. In addition, the majority of cardiac imaging results was solely obtained by
echocardiography in this study. Future studies employing more advanced cardiac imaging methods such as Magnetic resonance imaging (MRI) and PET imaging will warrant more accurate and sophisticated cardiac analyses. Lastly, this approach necessitates somewhat complicated technical and surgical procedures to successfully inject hiPSC-CMs and implant hMSC-PA to the heart. Follow-up studies should be carried out to develop a more concise surgical method that can take full advantage of this approach with less invasive procedures.

In summary, we report a strategy for cardiac repair which can concurrently rejuvenate both the myocardium and vasculatures through two major types of stem cells. Epidemic patch carrying hMSCs improved vascular regeneration and promoted engraftment and viability of the injected hiPSC-CMs leading to subsequent restoration of cardiac function. Our study highlights the unique advantages of different cell types, and their appropriate use can significantly advance cell-based cardiac therapy.

Methods

Mesenchymal stem cells derived from human bone marrow. Human mesenchymal stem cells derived from the bone marrow (hMSCs; Catholic Mater- TER Cells) were obtained from Catholic Institute of Cell Therapy (CIC, Seoul, Korea). Human bone marrow aspirates were obtained from the iliac crest of healthy donors aged 20 to 55 years after approval by the Institutional Review Board of Seoul St. Mary’s Hospital (approval numbers KIRB-00344-009 and KIRB-00362-006). We have complied with all relevant ethical regulations for work with human participants and obtained informed consent. Bone marrow aspirate from each consented donor was collected and sent to the GMP-compliant facility of Catholic Institute of Cell Therapy (Seoul, Korea, http://www.cic.re.kr) for the isolation, expansion, and quality control of hMSCs. The marrow mixture was centrifuged at 4 °C, 793 g for 7 min to obtain a marrow pellet. After removal of the supernatant, red blood cells were removed by adding and suspending in tenfold volume of sterile distilled water. Cell pellet obtained by centrifugating the RBC-deprived sample, was then suspended in the MSC growth medium (Dulbecco’s modified Eagle’s medium-low glucose (DMEM-LG, PAA), 10% fetal bovine serum (FBS, Gibco)). They were added to 100-mm tissue culture dish (TTC), which was then kept at 37 °C for 24 h for additional thermal cross-linking. After 48 h, the medium was changed daily, and hMSCs were allowed to grow in mTeSRi for 3–4 days until the cells were 90% confluent. At day 0, cells were treated with CHIR99021 (Tocris) 8 µM in CDM (cardiomyocyte differentiation medium) RPMI1640 (ThermoFisher Scientific)/BSA (Sigma-Aldrich)/ascorbic acid (Sigma-Aldrich). After 48 h, the medium was changed to CDM supplemented with 3 µM/ml CS9 (Wnt inhibitor/Stemgent Inc.) for another 48 h9–11. At day 5, the medium was replaced with CDM and freshly changed every 2 days. Spontaneously, contracting cells began to appear at ~day 6 to day 10. From day 10 to day 15, the medium was replaced with CDM containing L-lactic acid to metabolically select and purify cardiomyocytes (hiPSC-CMs)12. All live images were taken with a Lumascope 720 microscope (Etluma).

Generation of hiPSC-CMF. hiPSCs expressing GFP signal (hiPSC-GFP) was generated by using pMXs-EGFP retrovector. Briefly, the pMXs-EGFP retrovector was purchased from Addgene and transfected into the 293FT cells using the chemical method, Lipofectamine 2000 (Invitrogen). After 48 h, the virus-containing supernatants were collected filtered through a 0.45-µm filter (Millipore), and then concentrated using Retroconcentin (SBI: Mountain View, CA). Subsequently, these were transfected to 5.0 × 10^5 of hiPSC (DF19–9–11T) in opti-MEM media (Life Technologies) supplemented with 4 mg/mL polybrene (Millipore). Finally, hiPSC colonies expressing GFP signal were FACs sorted based on the expression of EGFP at day 10, to isolate hiPSCs-GFP (Supplementary Fig. 9). The purified fraction of hiPSC-GFP colonies were expanded for further differentiation into the CMs. Finally, hiPSC-GFP were differentiated into the hiPSC-CMs-GFP through the previously used CM differentiation protocol.

Myocardial infarction model and cell/patch delivery. All animal studies were approved by Animal Care and Use Committee in the Catholic University of Korea. We have complied with all relevant ethical regulations for animal testing and research. Fischer 344 rats (180–200 g, male, Orientbio, Korea) were anesthetized with 2% inhaled isoflurane and intubated via the trachea with an 18-gauge intravenous catheter. The rats were then mechanically ventilated with medical grade oxygen. Animals were placed on a 37 °C heating pad to prevent cooling during the surgical intervention. After shaving the chest, a thoracotomy was performed. MI was achieved by tying a suture with sterile polyethylene glycol tubing (22 G) placed into the left anterior descending (LAD) artery for 1 min, and then the knot was permanently ligated using a 7–0 prolene suture. To establish baseline left ventricular function, the ejection fraction (EF) and regional wall motion abnormalities (RWMA) were examined post operation day (POD) 7 (inclusion criterion: EF <45% by echocardiographic evaluation). On the same day, rats were anesthetized again using isoflurane inhalation, intubated and mechanically ventilated. The animal chest was re-opened, and the pericardium was partially removed from the infarcted heart. Then, hiPSC-CMs (1.0 × 10^6 per rat) were injected at two different sites: either the lower zone of infarcted myocardium and the hMSC-PA were implanted directly on the epicardium using two sutures (Supplementary Movie 3). To trace the injected hiPSC-CMs within the heart tissues, we used CM-Dil (Chloromethylenobenzimidazo, CellTracker TM). The stock solution was prepared using a modification of the manufacturer’s instructions. From a 1 mg/ml CM-Dil stock solution in DMSO, 5 µM solutions were made in 300 µl of the DMEM and this working solution was used to label the hiPSC-CMs. The chest was closed aseptically, and antibiotics and 0.9% normal saline solution was given. All rats received following immunosuppressants (azathioprine, 2 mg/kg; cyclosporine A, 5 mg/kg; methylprednisolone, 5 mg/kg) daily.

Echocardiography. The assessment of functional improvement for injured cardiac tissues was performed with echocardiography26,33,34. The rats were lightly anesthetized with inhaled isoflurane, and physiological data were recorded by using Transonic echocardiography system equipped with a 15 MHz L15–70 linear transducer (Affiniti 50 G, Philips). Serial echocardiograms were performed at 2, 4, and 8 weeks after treatment. The echocardiography operator was blinded to the group allocation during the experiment. Ejection fraction (EF) and fractional shortening (FS), which are indices of LV systolic function, were calculated with the following equations, respectively:

\[ EF(\%) = \left( \frac{LVEF_{ED}}{LVEF_{ES}} - 1 \right) \times 100 \]  

\[ FS(\%) = \left( \frac{LVEDD - LVEDV}{LVEDV} \right) \times 100 \]

Flow-cytometry analysis. hMSCs were re-suspended in 100 µl of FACS solution (1% FBS in PBS) then incubated with PE-conjugated mouse anti-human 1:250 (BD Biosciences # 555956), 1:250 CD73 (BD Biosciences # 550257), 1:250 CD105 (BD Biosciences # 560839), and APC-conjugated mouse anti-human 1:250 CD105.
Quantitative real-time RT-PCR. The total RNAs were extracted by the addition of 0.5 mL of TRIzol reagent (Life Technologies) to cells on a plate as described in the manufacturer’s instructions. One microgram of RNA was subjected to cDNA synthesis with SuperScriptTM Reverse Transcriptase IV and random primers (Invitrogen) for 1 h at RT in the dark with secondary antibody 1:1000 Alexa Fluor 488 goat anti-mouse IgG (Thermo Fisher #A11008) and 1:1000 Alexa Fluor 555 goat anti-mouse IgG (Thermo Fisher #A21422) antibodies for 1 h at RT. All cells were analyzed using a FACSCalibur, Cell Quest software (BD Biosciences) with the exception of hiPSC-GFP which was sorted through a SH8005 Cell Sorter flow cytometer with Cell sorter software Ver 2.1.2 (Sony Biotechnology).

Determination of fibrosis. Masson’s Trichrome (MT) staining (Sigma) was performed to determine the fibrosis area of MI hearts. Briefly, three frozen sections were fixed in Bouin’s solution at 50°C for 15 min in each group. These sections were stained using Weigert’s iron hematoxylin solution for 5 min at room temperature and also stained using Biebrich Scarlet-acid Fuchsin solution for 2 min at room temperature. Finally, the sections were counterstained with Aniline Blue for 15 min at room temperature. Hearts were then fixed in Bouin’s solution for 20 min at 4 °C overnight before embedding in OCT compound (Thermo Scientific) with dry ice. In all, 10-μm cross-sections of the heart were made by using HM525 NX Cryostat (Thermo Scientific) starting from the apex to the top. The sections were stored in −80°C before use. The number of capsules were counted in five random microscopic fields using a fluorescence microscope (Nikon) and expressed as the number of capsules per square millimeter tissue.

Immunocytochemistry. Cells were plated onto gelatin-coated glass dish and cultured for 5 days. Then cells were fixed with 4% PFA for 20 min at 4°C, permeabilized with 0.1% BSA in 0.03% Triton X-100 for 30 min at RT. Subsequently, the cells were stained with 1:100 CD90 (Abcam #Ab225), 1:500 TNNT2 (Thermo Fisher #MA5–12960), 1:200 ACTN4 (Sigma–Aldrich #A7811) IgG antibodies for 12 h at 4°C. Secondary staining was performed with antibody 1:100 Alexa Fluor 488 goat anti-rabbit IgG (Thermo Fisher #A21283), 1:100 Alexa Fluor 555 goat anti-mouse IgG (Thermo Fisher #A21422) antibodies for 1 h at RT. All cells were analyzed using a Laser Scanning Microscope LSM 880 NLO with Airyscan processing (Zeiss).

Multi-electrode array measurements. In vitro co-cultures of NRVM and hiPSC-CMs were prepared. NRVMs and hiPSC-CMs were mixed at a ratio of 8 to 2 and seeded to a multi-electrode array (MEA) chamber (60MEA200, Multi Channel Systems, Reutlingen, Germany). Electrophysiological recording was performed 7 days after seeding. During the measurement, CM was placed in the MEA recording apparatus (MEA2100-System, Multi Channel Systems) maintained at 37°C and 5% CO₂. Spontaneous electrical activity of CM was recorded for 20 min. The recording and analysis of conduction velocity was performed and calculated with CardioD2 program (Multi Channel Systems).

TUNEL assay. TUNEL staining was performed via an In situ Apoptosis Detection kit (Invitrogen), as the manufacturer instructed. After TUNEL staining, heart sections were stained with antibodies specific for CMs or hMSCs. Samples were imaged using a confocal microscope (Zeiss). Images were randomly selected from each section to quantify the number of TUNEL-positive cells.

Production of hMSC-conditioned medium. hMSCs (2 × 10^5) were seeded onto 100-mm dishes, and were cultured until 80-90% confluency. Cells were then washed with PBS, and the medium was changed to low glucose DMEM (Lonza) without FBS. After 7 of 14 days of culture, the supernatants were collected and kept at 4°C for further experiments.

Tube-formation assay. Basement membrane matrix (Matrigel®, BD Biosciences) was added to two-well chamber slides and solidified by incubation at 37°C for 30 min. Overall, 1 × 10^4 human umbilical vein endothelial cells (HUVECs) were plated onto each Matrigel-containing well with DMEM/F12 medium containing 20% KnockOut serum replacement, 1% nonessential amino acids, 0.1 mM β-mercaptoethanol, 4 mM HEPES, 10 ng/mL VEGF, 10 ng/mL EGF, and 25 ng/mL DLL4 and incubated at 37°C for 12 h. After removing the media, 4% PFA was added for fixation. The tube structures were evaluated by microscopy.

Endothelial cell-migration assay. HUVEC (3.5 × 10^4 cells/well) were cultured in a well separated by a Culture-Insert 24 (ibidi, Martinsried, Germany). Next, to record the migration of cells to the cell-free gap, the culture inserts were removed after 24 h. Cells cultured in ERM-2 media (Lonza) supplemented with 10% hMSC-conditioned media for another 18 h. Subsequently, the migration area was digitally photographed during the indicated hours (0, 6, 12, and 18 h) and area of the cell-free gap was calculated by using ImageJ software (version 6.0, NIH). Migration rate was determined and expressed as a percentage of closure relative to the initial size at 0 h. All images were taken with a Lumampe 720 microscope (Etlam) in time-lapse at 5 min intervals.

Endothelial cell proliferation assay. A co-culture system involving hMSCs and HUVECs (Lonza) was used to evaluate the proliferation of HUVECs. The proliferation rate of HUVEC droplets (1 × 10^4 cells; 10 μl) co-cultured with BM-MSCs were measured. Briefly, a total of 2 × 10^5 hMSCs were seeded in the upper layer of 35-mm plates and cultured with 2% FBS in the DMEM medium. After 4 h, HUVEC droplets (1 × 10^4 cells; 10 μl) were carefully placed into the bottom layer of each well. The HUVEC droplets were allowed to adhere at 37°C for 3 h followed by the addition of medium. Subsequently, H&E staining was carried out to measure the size of HUVEC droplet. Lastly, the total number of HUVECs per plate was quantified. Both HUVEC droplet size and cell number were quantified for 3 consecutive days.

Cytotoxic effects of hMSCs conditioned medium. To investigate whether hMSCs conditioned medium could provide cytoprotective effects against ischemic insult, 10% hMSC-conditioned medium was added into hiPSC-CM culture containing hydrogen peroxide (H_2O_2) (200 μM), simulating conditions of myocardial ischemia in vitro. Following 2 h exposure to H_2O_2, both hiPSC-CMs and culture medium were harvested. First, hiPSC-CMs were examined by using Annexin V–FITC kit (Biolgend) to measure the apoptosis, and the results were analyzed by Aria® flow cytometer (SH800, Sony Biotechnology, Inc., Tokyo Japan) with sony software 2.1.2 version. In addition, lactate dehydrogenase (LDH) assay (LDH cytotoxicity assay kit, Sigma) was performed by using culture medium to measure cellular damage. Briefly, 50 μl of culture medium was collected from various groups of hiPSC-CM cultures (in triplicate) and transferred into a 96-well plate. After adding an equivalent volume of LDH reagent into each well, the absorbance was measured using a spectrophotometer at a wavelength of 492 nm with a reference wavelength of 620 nm. For more accurate measurements, the absorbance of the no-cell controls was subtracted from the readings of hiPSC-CM samples.

Cardiomyocyte migration assay. hiPSC-CMs and hMSCs were cultured on Culture-Insert 2 well (ibidi) at 2 × 10^4 cells/well. The culture inserts were removed.
after 36 h. Both cell types were cultured in the RPMI medium supplemented with 2% FBS for 48 h at 37 °C. The migration area was digitally photographed at the indicated hours (0, 12, 24, and 48 h). All images were taken with a LumaScope 720 microscope (Etaluma) in time-lapse videos at 3 min intervals.

Cytokine array. hMSC-conditioned medium was analyzed by using biotin label-based human antibody array I membrane for 507 human proteins (RayBiotech, Norcross, GA).13,37.

Statistical analysis. All quantitative data are shown as means ± SE unless otherwise indicated. The statistical differences between two groups were analyzed by two-tailed Student’s t tests. The Statistical differences among three or more groups were analyzed by one-way ANOVA with Bonferroni’s post hoc analysis. The results were considered statistically significant when the p-value was less than 0.05.

Data availability

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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