

1 **Therapeutic potential of human iPS cell-derived cardiac tissue in an ischemic model**
2 **with unloaded condition mimicking left ventricular assist device**

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Conflict of Interest Statement

J.K.Y. is a founder, equity holder, and scientific adviser of iHeart Japan Corporation. J.

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Animal experiments

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53

54 **Glossary of Abbreviations:**

55 CTSs = cardiac tissue sheets

56 hiPSC = Human induced pluripotent stem cell

57 HiCT = hiPSC-derived cardiac tissue

58 HTx = heterotopic heart transplantation

59 ICM = ischemic cardiomyopathy

60 LVAD = left ventricular assist device

61 MI = myocardial infarction

62 MuRF-1 = muscle-specific RING finger 1

63 PCM1 = pericentriolar material 1

64 SERCA2a = sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase 2a

65 VAD = ventricular assist device

66

67 **Central picture legend:**

68 HiCT transplant on unloaded heart reduces infarct remodeling and promotes graft
69 survival.

70

71 **Central message:**

72 HiCTs transplantation to ischemic hearts under unloading condition promotes graft
73 survival, attenuates infarct remodeling, promotes neovascularization and prevents
74 atrophy of cardiomyocytes.

75

76 **Perspective Statement:**

77 HiCT treatment holds potential as an effective treatment for ischemic hearts supported by
78 ventricular assist devices (VADs). It can promote graft survival, reduce infarct
79 remodeling, stimulate neovascularization, and attenuate cardiomyocyte atrophy. These
80 findings suggest that HiCT treatment could serve as a "bridge to recovery" strategy for
81 VAD patients.

82

ABSTRACT

Objective: This study aimed to explore the therapeutic potential of hiPSC-derived cardiac tissues (HiCTs) in the emerging approach of "Bridge to recovery (BTR)" for severe heart failure with ventricular assist devices (VADs). We utilized a rat model of heterotopic heart transplantation (HTx) to mimic VAD support and heart unloading.

Methods: HiCTs were created by inserting gelatin hydrogel microspheres between cell sheets made from hiPSC-derived cardiovascular cells. Male athymic nude rats underwent myocardial infarction (MI) and were divided into the following groups: MI (loaded, untreated control), MI+HTx (unloaded, untreated control), MI+HTx+HiCT (unloaded, treated), and MI+HiCT (loaded, treated). HiCTs were placed on the epicardium of the heart in treated groups. We evaluated HiCT engraftment, fibrosis, neovascularization using histological analysis.

Results: After four weeks, HiCTs successfully engrafted in five out of six rats in the MI+HTx+HiCT group (83.3%). The engrafted HiCT area was greater under unloaded conditions (MI+HTx+HiCT) than loaded conditions (MI+HiCT) ($P<0.05$).

99 MI+HTx+HiCT had a significantly smaller infarct area compared to MI and MI+HTx.

100 The MI+HTx+MiCT group exhibited higher vascular density in the border zone than MI

101 and MI+HTx. HiCT treatment suppressed cardiomyocyte atrophy due to LV unloading

102 (P=0.001). The protein level of MuRF1, an atrophy-related ubiquitin ligase, was lower in

103 the MI+HTx+HiCT group than MI+HTx (P=0.036). However, HiCT treatment did not

104 significantly improve LV systolic function in unloaded hearts.

105 Conclusions: Transplanting HiCTs into ischemic hearts under unloaded conditions

106 promoted engraftment, neovascularization, attenuated infarct remodeling, and suppressed

107 myocyte atrophy. These results suggest that HiCT treatment could contribute to future

108 advancements in BTR. (250 words)

109
110 **Key words:**

111 Ventricular assist device; stem cell; bridge to recovery.

INTRODUCTION

Cardiovascular disease continues to be the primary cause of death globally¹. Within this category, ischemic heart disease (IHD) is responsible for most common cases of heart failure with reduced ejection fraction^{2, 3}. While heart transplantation is an effective treatment for patients with end-stage heart failure who are unresponsive to other therapies, the scarcity of heart donors limits the widespread application of this therapeutic option for the majority of individuals suffering from severe heart failure.

One of the palliative therapies for end-stage heart failure is the use of a ventricular assist device (VAD)⁴. The implantation of a VAD allows heart failure patients to survive by maintaining systemic circulation. Clinically, VADs have been employed as a bridging measure for heart transplantation while patients wait for a suitable donor (bridge to transplantation), as well as a lifelong circulatory support (destination therapy)⁵.⁶ Additionally, recent investigations have focused on "bridge to recovery (BTR)", which is another option for utilizing VADs. BTR involves a multidisciplinary approach aimed at anticipating functional recovery of the failing heart by combining VAD support.

Studies in animal models^{7, 8} and clinical settings⁹ have reported that left ventricular (LV) unloading through VADs can restore cardiac gene expression and reduce LV dilatation, along with other biomedical interventions.

In research aimed at achieving BTR, attempts have been made with gene therapy or stem cell transplantation^{10, 11}. It is assumed that the combination therapy of VAD therapy and cell transplantation not only provides an additive effect through LV unloading with the VAD therapy and the paracrine effect of cell therapy but also offers a synergistic effect where mechanical unloading creates a favorable condition for cell transplantation. It has been reported that the unloading condition improved the engraftment efficiency of cardiac stem cells in an infarcted heart during mouse heterotopic heart transplantation (HTx) model¹². However, the reported clinical trials of BTR utilizing stem cell transplantation have so far fallen short of expectations in terms of the success rate of the strategy, including functional recovery and weaning from VAD support^{6, 13, 14}.

In our previous studies, we reported that transplanting human induced pluripotent stem cell (hiPSC)-derived cardiac tissue sheets (CTSs) containing

cardiomyocytes, vascular endothelial cells, and vascular mural cells into rat and pig subacute myocardial infarction (MI) models resulted in improved cardiac function by attenuating infarct remodeling and promoting angiogenesis^{15, 16}. Additionally, we observed a higher therapeutic and myocardial regenerative effect in epicardial transplantation of human iPS cell-derived cardiac tissue (HiCT) onto a rat MI model¹⁷, which involved layering CTSs using gelatin hydrogel microspheres (the gelatin hydrogel microsphere layer and the cell layer exist adjacent to each other rather than being mixed) to enhance oxygen and nutrient supply within the thick tissue^{18, 19}. Transplanting HiCT onto patients undergoing VAD therapy may hold promise as a new strategy for bridge to recovery (BTR).

In this study, we examined the impact of transplanting HiCT onto an unloaded rat heart, mimicking VAD support, and evaluated the potential for bridge to recovery (BTR) in the experimental model. Additionally, we assessed the biological effects of HiCT treatment on the unloaded heart, including graft survival and infarct remodeling.

MATERIAL AND METHODS

Detailed methods are provided as Supporting Information online.

Experimental protocol

All experimental procedures were carried out in accordance with the animal care guidelines established by Kyoto University and the "*Guide for the Care and Use of Laboratory Animals*" published by the National Institutes of Health. All animal experimental protocols were approved by the Animal Experimentation Committee of Kyoto University (#Med Kyo 15261).

The time course of the experiment is illustrated in Figure 1A. The rats that successfully developed myocardial infarction (MI), characterized by fractional shortening less than 40%, were divided into the following groups: (1) MI group (n=6) (loaded, untreated control) underwent no HTx. A laparotomy was performed, and the abdominal aorta was exposed as a sham operation for HTx. (2) MI+HTx group (n=31) (unloaded, untreated control) underwent HTx. Sixteen rats survived after HTx. (3) MI+HTx+HiCT group (n=28) (unloaded, treated) underwent HTx followed by HiCT treatment. Seventeen

rats survived after HTx. For the comparison of engraftment of HiCT with or without unloading, we added a group as follows: (4) MI+HiCT group (n=5) (loaded, treated). They did not undergo HTx but received HiCT treatment. The comparisons are explained in Figure 1B and Figure 5A. The experimental group is presented in Supplemental Figure 1.

Human iPSC-derived cardiac tissue (HiCT) formation

The cardiovascular cells differentiated from hiPSC line (201B6) were dissociated and then plated onto a 10% fetal bovine serum (FBS)-coated 12-multiwell UpCell (CellSeed, Tokyo, Japan) at a density of $10-12 \times 10^5$ cells per well. After 4 days in culture, the cells were transferred to room temperature to allow for the detachment of monolayer cell sheets. The size of the collected cell sheets was almost identical in every experimental repeats. It was then plated onto a Matrigel-coated 6-cm dish, and 0.5 mg of gelatin hydrogel microspheres^{17, 18} dissolved in phosphate-buffered saline (PBS) was placed on the surface of the cell sheet. After 45 minutes, another monolayer cell sheet was stacked on top of

the previous one. This stacking process was repeated 4 times, resulting in a five-layered cell sheet known as human iPSC-derived cardiac tissue (HiCT) (Figure 1C).

Flow cytometry

The monolayer cell sheet was dissociated and then stained with surface markers specific for each cell lineage. The cellular components of monolayer cell sheets before preparation of the HiCTs included 52.0 ± 4.9 % of cardiac isoform of troponin-T (cTnT)-positive cardiomyocytes, 6.5 ± 2.2 % of vascular endothelial (VE)-cadherin-positive vascular endothelial cells, 8.8 ± 4.9 % of platelet-derived growth factor receptor beta (PDGFR β)-positive vascular mural cells and 0.3 ± 0.1 % of TRA-1-60-positive undifferentiated cells, respectively (Figure 1D).

Subacute MI rat model

Male athymic nude rats (F344/N Jcl-rnu/rnu, CLEA Japan, Inc., Tokyo, Japan) aged 12-17 weeks were utilized as donors and recipients for transplantation, respectively. The MI model rats were generated following previously described methods¹⁵.

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210 **Heterotopic heart transplantation and HiCT treatment**

211 Heterotopic heart transplantation was performed following previously established
212 methods²⁰. Briefly, one week after creating the MI, the donor rat was anesthetized, the
213 right and left superior vena cava and IVC were ligated, and 5 mL of cold cardioplegia
214 solution (Miotector; Mochida, Tokyo, Japan) were injected from the ascending aorta for
215 cardioprotection. Once cardiac arrest was achieved, the infarcted heart and lung were
216 harvested and preserved in cold cardioplegia solution. Next, the recipient rat was
217 anesthetized and underwent laparotomy. The ascending aorta of the donor heart was
218 anastomosed end-to-side to the recipient rat abdominal aorta. After reperfusion, the
219 transplanted heart spontaneously resumed beating immediately. In the MI+HTx+HiCT
220 group, the HiCT was placed on the epicardium of the MI region and manually spread to
221 cover the entire area of the infarction (Figure 1E).

222

223 **Histological analysis**

224 Hearts were harvested 4 weeks after HTx (5 weeks after MI induction). The animals were
225 anesthetized with 1% isoflurane while placed on a volume cycled ventilator for small
226 animals. After opening the chest and abdomen, 100 ml of heparinized cold saline (500
227 IU) followed by 50 mL of 4% paraformaldehyde (PFA) were infused through the apex of
228 the recipient's heart. The heterotopically transplanted heart in the abdomen was rapidly
229 excised and placed in 4% PFA overnight. Afterward, it was embedded in OCT compound
230 (Sakura Finetek Japan, Tokyo, Japan) and frozen. The tissue was transversely sliced into
231 7 µm sections just below the ligation point and subjected to Hematoxylin-Eosin and
232 Masson trichrome staining, as well as immunofluorescence staining. For
233 immunofluorescence staining, the sections were treated with Protein Block Serum Free
234 (DAKO, Glostrup, Danish) and incubated overnight with primary antibodies at 4°C. Anti-
235 mouse Alexa 546 (1:500) and anti-rabbit Alexa 488 (Invitrogen, Eugene, OR, USA)
236 (1:400) were used as secondary antibodies. The area of engrafted human cells was
237 manually traced as positive cell clusters, identified by staining with anti-cTnT antibody
238 (rabbit polyclonal; Abcam, Cambridge, UK; 1:500) and human nuclear antibody (HNA)
239 (mouse monoclonal, clone 235-1; Millipore, Billerica, MA, USA; 1:200). To evaluate the

240 proliferation of transplanted cardiomyocytes, double-positive cells stained with anti-Ki67
241 antibody (rabbit monoclonal, clone D3B5; Cell Signaling Technology, Danvers, MA,
242 USA) and anti-cTnT antibody (rabbit polyclonal antibody; Thermo Fisher Scientific,
243 Waltham, MA, USA; 1:500) were visualized. The length of fibrotic lesions at the
244 endocardium level and the total length of the endocardium were manually traced and
245 measured using Masson's trichrome-stained sections, and the ratio of the MI length to the
246 total length was calculated for each section. The MI area was measured as the ratio of the
247 area of fibrotic lesions to left ventricular myocardium. The area of fibrotic lesion and left
248 ventricular myocardium were manually traced and measured using Masson's trichrome-
249 stained sections. We established a standardized criterion by preparing one section at the
250 same location for each rat, specifically just below the permanent ligation thread of the
251 coronary artery introduced during MI induction. Wall thickness was calculated as the
252 average of five randomly selected vertical distances of the wall (including both ends) in
253 the MI area. For vascular density (number of vessels/mm²), four views of the border zone
254 (two views on each side) were selected from vWF-stained sections, and the number of
255 vessels was manually counted in each view. A rabbit polyclonal vWF antibody (DAKO;

1:1000) was used as the primary antibody for vWF staining. A mouse monoclonal cTnT antibody (clone 13211, Thermo Fisher Scientific; 1:500) was used for double staining with cTnT and vWF. To measure the size of cardiomyocytes, five views of the non-infarcted area in the left ventricle were selected from Pericentriolar Material 1 (PCMI) and eosin-stained sections. The size of cardiomyocytes was calculated from randomly selected 50 cardiomyocytes (10 cardiomyocytes in each view) whose nuclei were stained with anti-PCMI antibody (a cardiomyocyte nuclear marker²¹) and whose cytoplasmic membrane appeared intact and had a round shape. For PCMI staining, a rabbit polyclonal antibody (Thermo Fisher Scientific; 1:400) was used as the primary antibody. Images were captured using an all-in-one digital microscope (BIOREVO BZ-9000; Keyence, Osaka, Japan) and analyzed using the BZ-X Analyzer (Keyence) software and ImageJ²² (U.S. National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

The data were analyzed using GraphPad Prism software for Mac (version 7.0, San Diego, California, USA). The results are presented as mean $\pm$ standard deviation. Comparisons

between two groups were performed using the unpaired t-test or Mann-Whitney test. Comparisons among three groups were carried out using one-way analysis of variance (ANOVA) followed by Tukey's test as a post hoc analysis. The correlation was assessed using Pearson's correlation coefficient. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Baseline surgical data

The baseline echocardiogram data prior to MI induction are presented in Supplemental Table 1. The survival rate following MI induction and subsequent heterotopic transplantation is illustrated in Supplemental Figure 1. As demonstrated in Supplemental Table 2, there were no significant differences in cardiac function among the three groups of rats enrolled in the study, as confirmed by echocardiography one week after MI. Additionally, there were no significant differences in the total operation time of whole host and recipient surgeries, operation time for recipient rats (transplantation time), and

the time from donor heart resection to reperfusion of the transplanted heart (ischemic time) (Figure 1F).

Engraftment after HiCT transplantation onto a rat MI model

Immunofluorescent staining revealed successful engraftment of transplanted HiCTs, covering the infarcted region in 5 out of 6 rats in the MI+HTx+HiCT group, four weeks after transplantation (Figure 2A,B). Almost all engrafted HiCTs consisted of HNA-positive human cells, with the majority being cardiac troponin-T (cTnT)-positive cardiomyocytes. Additionally, we observed a higher content of Ki67-positive cardiomyocyte nuclei in the engrafted HiCTs compared to the native myocardium of rats, even at 4 weeks after HiCT treatment (Figure 2C). We confirmed that proliferating cells remained on the epicardium and did not penetrate into the myocardium. These findings suggest that HiCTs are successfully engrafted after transplanted into unloaded hearts, and the engrafted cardiomyocytes retain their ability to proliferate in the absence of mechanical loading.

304 **HiCT transplantation for unloaded heart attenuated infarct remodeling after MI**

305 Subsequently, we assessed the extent of infarct remodeling following MI under each
306 condition. The hearts in the MI group exhibited fibrosis extension and thinning of the
307 infarcted wall five weeks after MI, indicating the progression of infarct remodeling
308 (Figure 3A). However, the ratio of MI length to total LV length was significantly reduced
309 in the MI+HTx+HiCT group compared to the other two groups [MI vs. MI+HTx vs
310 MI+HTx+HiCT: 33.69 ± 6.26 vs. 34.56 ± 8.49 vs. $22.43 \pm 5.51\%$; $P=0.032$ (MI vs
311 MI+HTx+HiCT), 0.021 (MI+HTx vs MI+HTx+HiCT)] (Figure 3B). The percentage of
312 MI area in whole LV area was significantly reduced in MI+HTx+HiCT group compared
313 to that in MI+HTx group [MI vs MI+HTx vs MI+HTx+HiCT: 21.99 ± 4.63 vs 28.08 ± 7.10
314 vs $19.05 \pm 4.05\%$; $P=0.028$ (MI+HTx vs MI;HTx+HiCT)] (Figure 3C). On the other hand,
315 the wall thickness of the MI+HTx group tended to be thicker and was significantly thicker
316 in the MI+HTx+HiCT group compared to the MI group [MI vs. HTx vs. MI+HTx+HiCT:
317 0.761 ± 0.129 vs. 1.295 ± 0.294 vs. 1.570 ± 0.562 mm; $P=0.063$ (MI vs MI+HTx), 0.005 (MI
318 vs MI+HTx+HiCT)] (Figure 3D). These results indicate that heterotopic transplantation

tends to preserve LV wall thickness following MI, and HiCT treatment under unloading conditions attenuates infarct remodeling after MI.

HiCT treatment under unloaded condition promoted neovascularization in border zone of MI

We previously have reported that the limited extent of infarct remodeling observed with HiCT treatment is due to the promotion of neovascularization in the border zone of the MI mediated by the engrafted HiCTs in loaded hearts¹⁷. Here, we examined the vascular density in the border zone following HiCT treatment. There was no significant difference in vascular density of the border zone between the MI group and the MI+HTx group. However, the MI+HTx+HiCT group exhibited a higher vascular density in the border zone compared to the other two groups [MI vs. MI+HTx vs. MI+HTx+HiCT: 12.8 ± 4.728 vs. 16.07 ± 1.684 vs. $70.54 \pm 58.35/\text{mm}^2$; $P=0.025$ (MI vs MI+HTx+HiCT), 0.035 (MI+HTx vs MI+HTx+HiCT)] (Figure 4A, B). Furthermore, there was a correlation between vascular density and the area of engrafted HiCTs in the MI+HTx+HiCT group ($R^2=0.756$, $P=0.0245$) (Figure 4C). These results suggest that HiCT treatment under

unloaded conditions promotes neovascularization in the border zone, and the extent of neovascularization might be mediated by the engrafted HiCTs. Additionally, we confirmed that neovascularization was also promoted in the adjacent region (Figure 4D) and within the engrafted HiCTs (Figure 4E), supporting the notion that neovascularization aids in the engraftment of HiCTs.

Enhancement of the engraftment efficiency of HiCT in the unloaded heart

Next, we examined how the unloaded condition affect the efficiency of the HiCT engraftment. We compared the efficiency of HiCT engraftment between under unloaded condition (MI+HTx+HiCT group) and under loaded condition (MI+HiCT group) (Figure 5A). In MI+HiCT group, only 3 out of 5 transplanted HiCT (60%) were engrafted 4 weeks after transplantation, while 5 out of 6 transplanted HiCT (83%) were engrafted in MI+HTx+HiCT group. The engrafted area in MI+HTx+HiCT group was significantly larger than that in MI+HiCT group (MI+HiCT vs MI+HTx+HiCT: 0.216 ± 0.229 vs $0.841 \pm 0.632 \text{ mm}^2$; $P=0.0498$) (Figure 5B,C). These results indicate that the unloaded condition is more desirable for the engraftment of HiCT than loaded condition. The

positive ratio of Ki-67 (Figure 5D) was equivalent with that of engrafted HiCTs in unloaded heart (Figure 2C), indicating that the proliferation of cardiomyocytes is not affected by the loaded condition.

HiCT treatment attenuates the atrophy of cardiomyocytes under unloaded condition

According to reports, long-term LVAD implantation has been found to cause atrophy of cardiomyocytes²³. Therefore, we conducted an investigation to examine how HiCT treatment under unloading conditions affects the progression of cardiomyocyte atrophy caused by unloading. As illustrated in Figures 6A and 6B, the cross-sectional area of cardiomyocytes in the non-infarcted area was significantly smaller in the MI+HTx group compared to the MI+HTx+HiCT group (MI+HTx vs MI+HTx+HiCT: 336 ± 72.2 vs $622 \pm 139 \mu\text{m}^2$; $P=0.001$). Previous studies have reported that atrophy of skeletal muscle and myocardium during unloading is partly attributed to increased degradation of muscle protein triggered by the activation of the ubiquitin-proteasome system during unloading²³,²⁴. Consequently, we examined the protein expression level of muscle-specific RING

finger 1 (MuRF-1), which is one of the ubiquitin ligases associated with muscle atrophy.

In the MI+HTx+HiCT group, the protein expression level of MuRF-1 was significantly lower than that in the MI+HTx group (MI+HTx vs MI+HTx+HiCT: 0.953 ± 0.734 vs 0.197 ± 0.185 ; $P=0.036$) (Figures 6C,D). These findings suggest that HiCT treatment under unloading conditions mitigated cardiomyocyte atrophy by suppressing the activation of MuRF-1 protein expression during unloading.

DISCUSSION

In this study, we demonstrated the therapeutic potential of HiCT treatment in a rat subacute MI model under unloaded conditions (Figure 7). This was achieved by attenuating infarct remodeling, which we previously reported to be mediated by the promotion of neovascularization under loaded conditions as well^{15, 16}. Furthermore, we observed a significantly greater efficiency in the engraftment of HiCTs under unloaded conditions as opposed to loaded conditions, which would further enhance the therapeutic mechanisms. These findings suggest that HiCT treatment may offer a potential solution

383 for BTR, and it could be a viable therapeutic approach to simultaneously perform
384 epicardial transplantation of HiCTs during VAD implantation aiming to BTR. The
385 unloaded hearts showing preserved MI wall thickness (Figures 3A, D) would also provide
386 an advantage for functional recovery through HiCT treatment compared to the loaded
387 condition. It is worth noting that weaning from VAD is rare and mostly observed in
388 younger patients with non-ischemic cardiomyopathy or those with acute conditions like
389 myocarditis, rather than patients with ischemic cardiomyopathy²⁵⁻²⁸. Therefore, HiCT
390 treatment could emerge as a novel approach to enhance BTR for ischemic
391 cardiomyopathy. However, it is important to note that we were unable to observe
392 significant improvement in LV function by the HiCT treatment because it was technically
393 demanding to evaluate cardiac function of the heterotopically implanted hearts located in
394 abdominal region which may hinder the realization of a complete BTR approach based
395 solely on this strategy. Furthermore, in order to translate the treatment approach in this
396 study into practical applications for clinical BTR, it is necessary to go beyond using
397 subacute myocardial infarction models like the one employed in this study. We may need
398 to verify the therapeutic effects by including models of chronic ischemic cardiomyopathy

with fully developed scar tissue, and non-ischemic cardiomyopathy. Additionally, a more extended period of observation after treatment would be required to further assess whether HiCT transplantation therapy can indeed contribute to the clinical BTR.

Regarding the engraftment efficiency of HiCTs, it was found that the unloaded condition yielded better results compared to the loaded condition. This aligns with a study by Kurazumi et al., who reported an increased efficiency of cell engraftment in a mouse model when cardiac stem cells were intramyocardially injected under unloaded conditions¹². In our study, we explored tissue sheet transplantation on the surface of the heart and demonstrated that the unloaded condition was favorable even for this method, as it led to improved engraftment of transplanted cells. We assume there are two reasons for this result. First, the transplanted cells may benefit from improved blood supply under unloaded conditions. Watanabe et al. conducted a study using a pig model of ischemic heart failure and reported that the implantation of temporary ventricular support device (IMPELLA®) improved blood flow to the coronary artery²⁹. This suggests that the presence of a circulatory support device creates a better host condition for cell transplantation. In chronic heart failure, systemic perfusion decreases due to low cardiac

415 function or underlying ischemic heart diseases affecting coronary arterial perfusion.

416 Therefore, it is assumed that the unloaded condition provides a more favorable

417 environment for the host heart, enhancing the efficiency of transplanted cell engraftment

418 through increased coronary blood flow facilitated by circulatory support. Here, we made

419 an intriguing discovery that neovascularization facilitated by HiCT treatment also

420 supported the engraftment of HiCTs. This was evidenced by the observation that the

421 engrafted area showed a correlation with the density of capillaries in the border zone

422 (Figure 4C). Moreover, we observed enhanced neovascularization in the adjacent region

423 and inside the engrafted HiCTs (Figures 4D,E). It is possible that the improved

424 engraftment of HiCTs not only attenuated infarct remodeling but also sequentially

425 promoted neovascularization, thereby enhancing the host condition for engraftment.

426 Another reason is that the removal of LV distension through unloading might create a

427 more favorable environment for cell transplantation. In a failing heart, the LV experiences

428 excessive pressure and volume, resulting in tension on the LV wall³⁰ which may hinder

429 the survival of the cells. In a state of cardiac overload, it is suggested that mitochondrial

430 production is promoted, leading to the activation of reactive oxygen species production

which may promote cell death³¹. It would be possible that by relieving the pressure and volume overload, the tension on the transplanted HiCT is reduced, leading to an improved engraftment efficiency of HiCT.

In our research, we discovered that HiCT treatment effectively reduced the atrophy of cardiomyocytes in the non-infarcted area under unloaded conditions. Some studies have previously reported that the implantation of a VAD in an unloaded condition can morphologically restore hypertrophic cardiomyocytes caused by cellular remodeling after myocardial infarction^{32, 33}. However, other reports have suggested that this change leads to the atrophy of cardiomyocytes under unloaded conditions²³. This notion is further supported by research showing the atrophy of cardiomyocytes in a normal goat heart after VAD transplantation³⁴. Additionally, another study found that the ventricular volume of a rat normal heart decreased by 60% one month after heterotopic heart transplantation³⁵. The underlying mechanism behind this atrophy is believed to be the activation of atrophy-related ubiquitin ligases, which are specific to skeletal and cardiac muscles. These ligases promote the degradation of cardiac proteins under unloaded conditions²³. In fact, studies have shown an increase in atrophy-related ubiquitin ligase expression in heterotopically

transplanted mouse hearts and human hearts equipped with LVADs after unloading³⁶. In our study, we found that the HiCT treatment onto unloaded hearts exhibited lower expression of MuRF-1, one of the atrophy-related ubiquitin ligases, compared to the hearts without HiCT treatment. These results indicate that HiCT treatment under unloaded conditions suppresses the atrophy of cardiomyocytes in the non-infarcted area by attenuating the protein expression of MuRF-1. This suppression of atrophy, a major drawback of long-term LVAD support, may increase the likelihood of successful BTR.

There are several limitations in the present study. First, in our current research, we attempted to replicate the physiological state of an unloaded heart supported by LVAD using a rat heterotopic transplantation model. However, this model does not fully replicate the cardiac physiology of the hearts supported by LVAD in a clinical context. To address this challenge, as the next step in our study, we believe it is necessary to assess the effects of HiCT transplantation in a setting more closely resembling the clinical use of LVAD, possibly by utilizing a LVAD implantation model in large animals. Second, due to technical complexities, we were unable to assess the function of the transplanted heart in this study. To explore the potential for BTR through HiCT transplantation, it would be

desirable to demonstrate an improvement in cardiac function with an unloaded heart mediated by HiCT transplantation, which can be considered a limitation in this study. In future experiments using large animals and LVAD implantation, rather than heterotopic transplantation, we plan to accurately evaluate how HiCT transplantation has affected cardiac function.

CONCLUSIONS

Transplanting HiCTs into ischemic hearts while in an unloaded condition has been shown to enhance neovascularization, reduce infarct remodeling, and prevent cardiomyocyte atrophy caused by unloading, a disadvantage associated with long-term LVAD implantation. Additionally, the engraftment efficiency of HiCTs was found to be higher under unloaded conditions compared to loaded conditions. These findings suggest that HiCT treatment has the potential to effectively treat ischemic hearts supported by LVAD and may serve as a strategy for achieving a BTR for VAD in the future.

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485 **Declaration of generative AI and AI-assisted technologies in the writing process**

486

487 During the preparation of this work the authors used ChatGPT in order to improve
488 language and readability, with caution. After using this tool/service, the authors reviewed
489 and edited the content as needed and take full responsibility for the content of the
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491

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597 **FIGURE LEGENDS**

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599 **FIGURE 1: Heterotopic heart transplantation and HiCT transplantation.**

(A) The time course of the experiments. (B) Schema of surgical groups. (C) Representative Macroscopic appearance of HiCT. Scale bar: 1 cm. (D) Cellular components of monolayer cell sheets before preparation of HiCTs (n=8). (E) Representative surgical appearance of HTx and HiCT transplantation. (F) Duration of surgeries. WB, Western blotting; MI, myocardial infarction; HTx, heterotopic heart transplantation; HiCT, human iPS-derived cardiac tissue sheets; CM, cardiomyocyte; EC, vascular endothelial cell; MC, vascular mural cell; cTnT, cardiac isoform of troponin-T; VE-cad, vascular-endothelial cadherin; PDGFRb, platelet-derived growth factor receptor beta.

FIGURE 2: Myocardial regeneration after HiCT transplantation onto unloaded hearts.

(A-C), Immunohistochemical analysis for engrafted HiCTs. (A) Representative immunostaining for HiCT on the surface of transplanted heart. HNA (red), cTnT (green) and DAPI (blue). Scale bars: 1 mm. (B) Higher magnification [dashed square in (A)]. Scale bars: 50 μ m. (C) Representative immunostaining for Ki67 (green), cTnT (red), and

DAPI (blue) for engrafted HiCT and native rat myocardium. White dotted lines indicate the margin of engrafted HiCT which was confirmed by consecutive sections stained with HNA and cTnT. Yellow arrows indicate Ki67 and cTnT double-positive cells. Scale bar: 100 μ m. HNA, human nucleic antigen; DAPI, 4',6-diamidino-2-phenylindole.

FIGURE 3: HiCT transplantation attenuates left ventricular remodeling after MI in unloaded hearts.

(A) Representative Masson's Trichrome staining of rat hearts 4weeks after HTx. Scale bars: 1 mm (B) The ratio of MI length of Total length. (C) The ratio of MI area to the total area of left ventricle. (D) Wall thickness.

FIGURE 4: Neovascularization after HiCT transplantation onto unloaded MI hearts.

(A) Representative immunostaining at 4 weeks after transplantation at border zone. vWF (green), cTnT (red), and DAPI (blue). Engrafted region of HiCT is not shown. Scale bars: 100 μ m. (B) Vascular number (diameter <100 μ m) per 1mm². (C) Correlation between

engrafted area and vascular density. **(D,E)** Representative immunostaining for neovascularization at adjacent region of HiCT **(D)** and inside of HiCT **(E)**; White arrow). White dotted line indicates the border of the engrafted region. Scale bars: 100 μ m.

FIGURE 5: Comparison of engrafted area of HiCT between loaded and unloaded MI hearts.

(A) Schema of surgical groups. **(B)** Representative immunostaining for HiCTs. HNA (red), cTnT (green) and DAPI (blue). Scale bars: 1 mm. **(C)** Quantitation of engrafted area. **(D)** Representative immunostaining for Ki67 (green), cTnT (red), and DAPI (blue) for engrafted HiCT and native rat myocardium. Yellow arrows indicate Ki67 and cTnT double-positive cells. Scale bars: 100 μ m.

FIGURE 6: Attenuation of cardiomyocyte atrophy after HiCT transplantation onto unloaded MI hearts.

(A) Representative histological staining of cardiomyocytes for PCM1 immunostaining and eosin. Black enclosed lines indicate single cardiomyocyte. Scale bars: 100 μ m. **(B)**

Cross section area of cardiomyocytes (n=50 each). (C) Representative Western blotting for MuRF-1. (D) MuRF-1/ β -actin protein level. PCM1, Pericentriolar material 1; MuRF-1, muscle-specific RING finger 1.

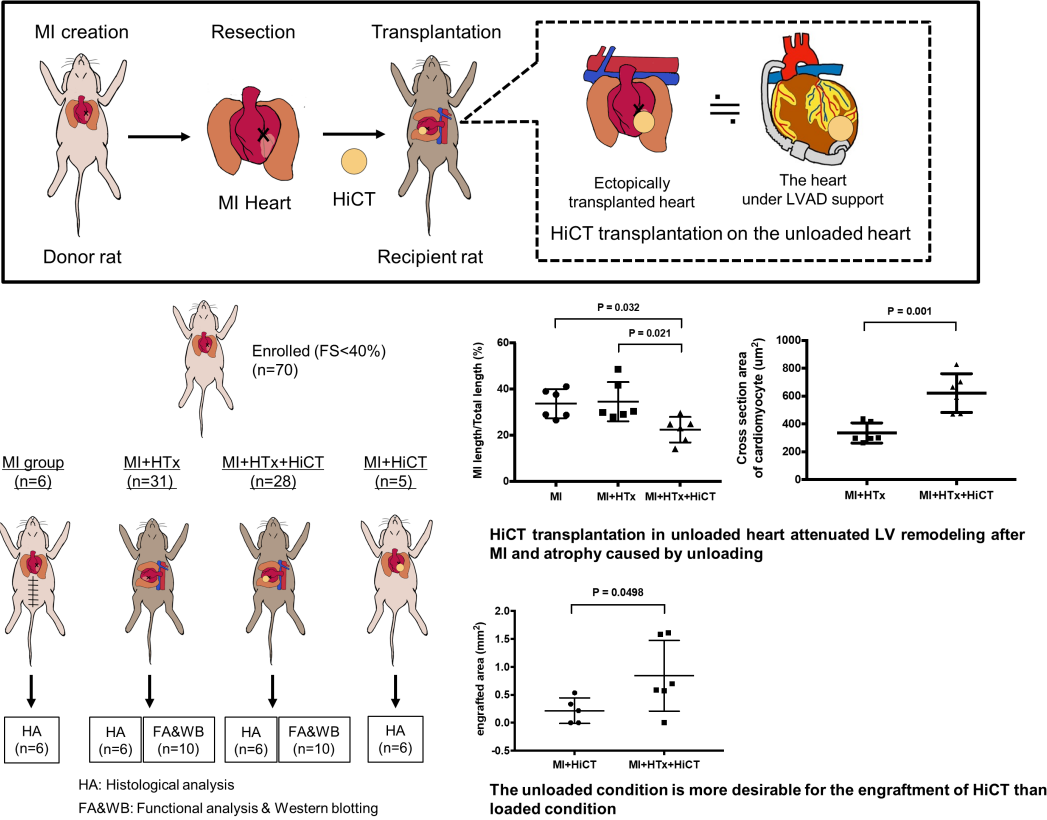
FIGURE 7: Schema of the research.

We utilized a rat model of heterotopic heart transplantation (HTx) to mimic VAD support and heart unloading, investigating the therapeutic potential of hiPSC-derived cardiac tissues (HiCTs) for severe heart failure with left ventricular assist devices (LVADs).

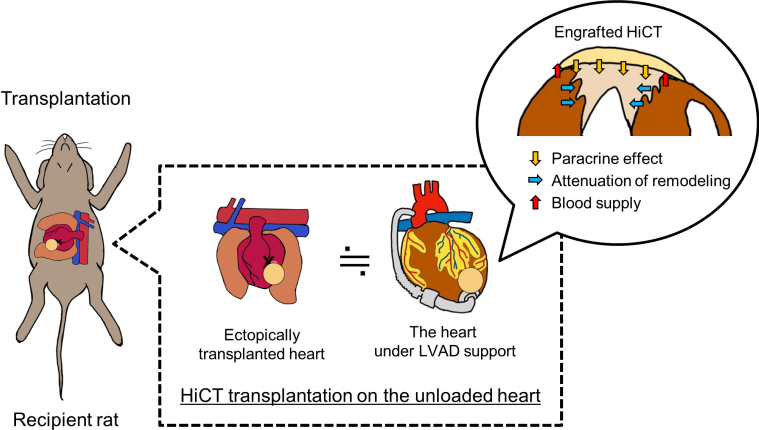
VIDEO LEGEND

VIDEO 1: Surgical procedure of the rat heterotopic heart transplantation model.

Graphical abstract



673 **Central picture**



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Figure1

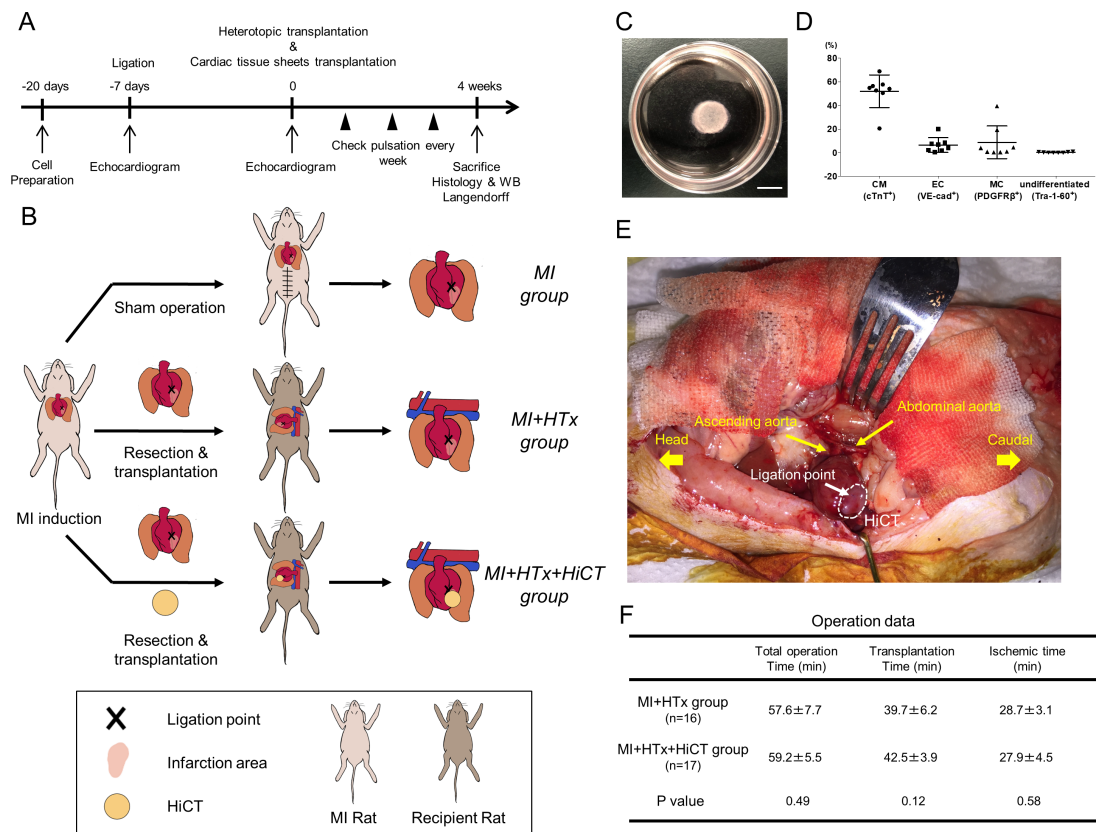


Figure2

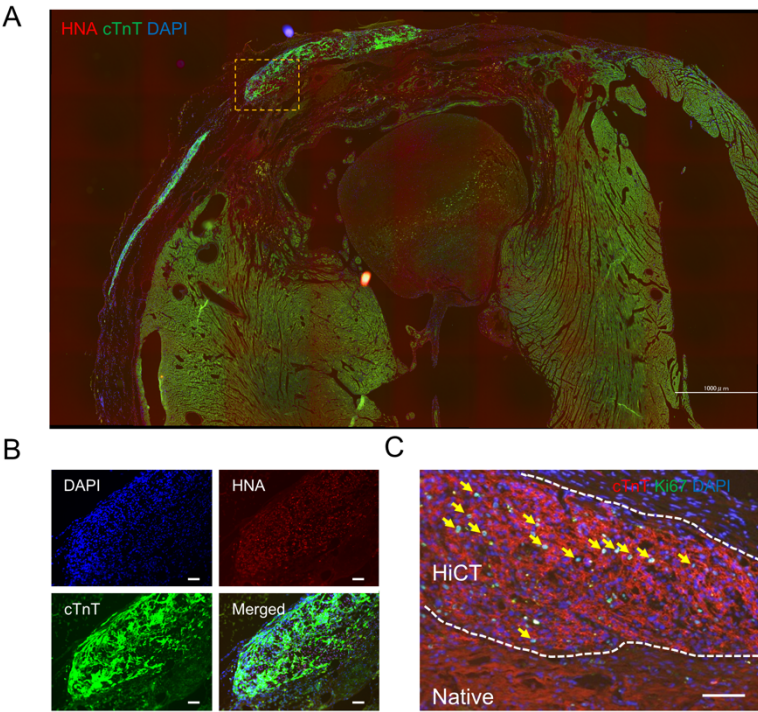
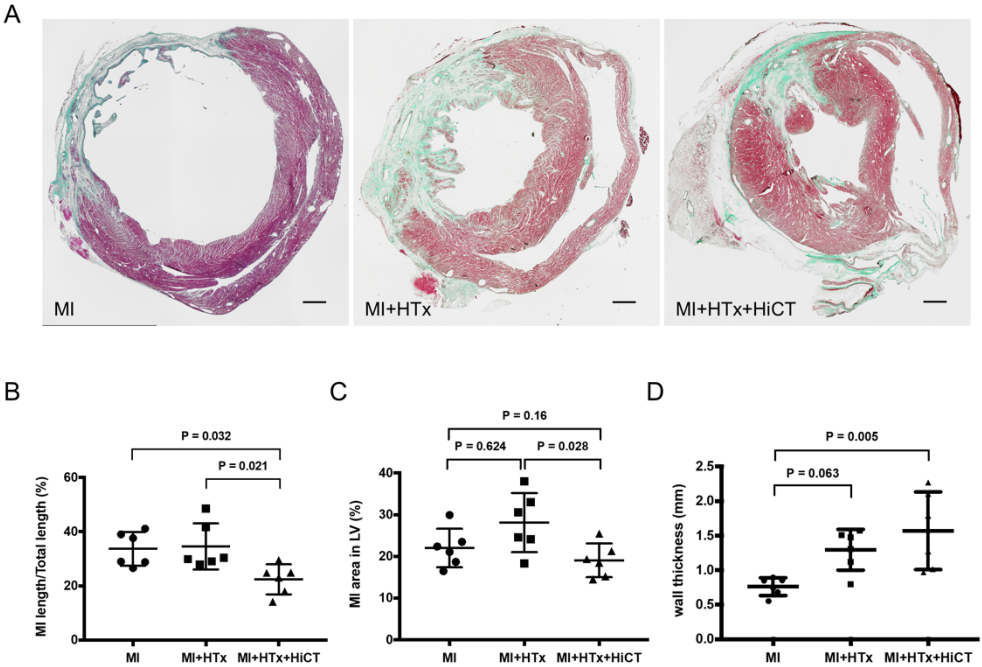
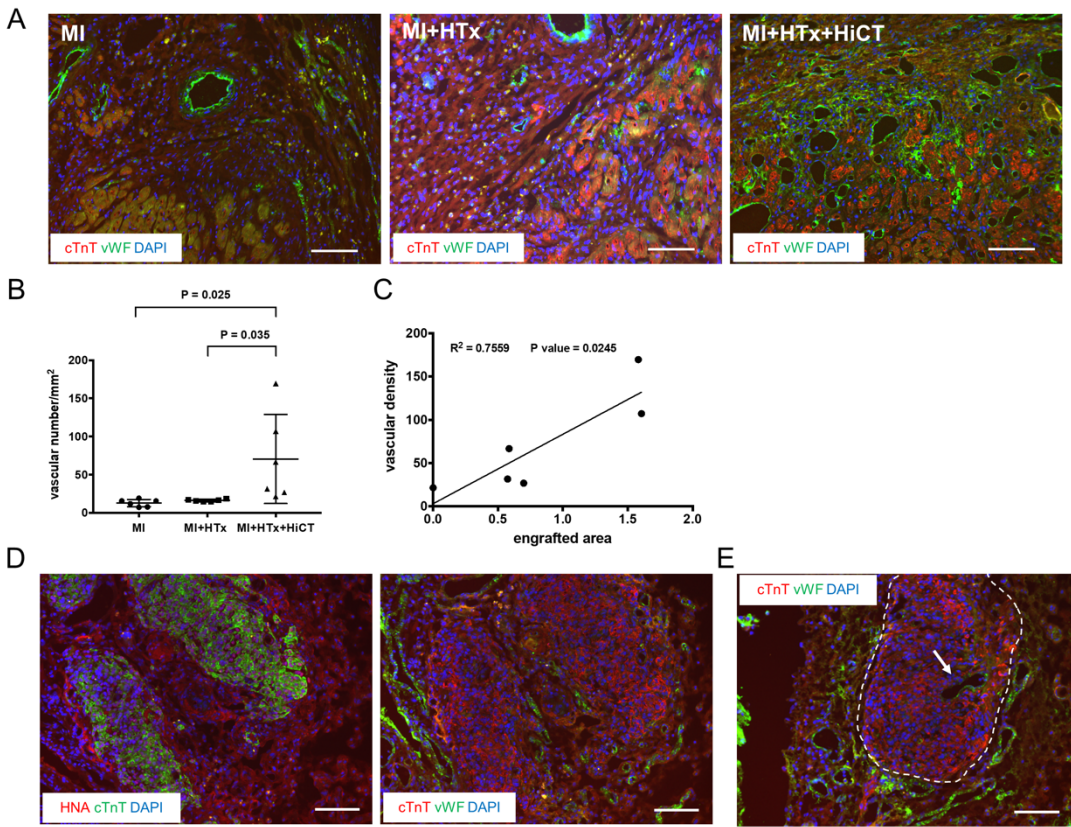


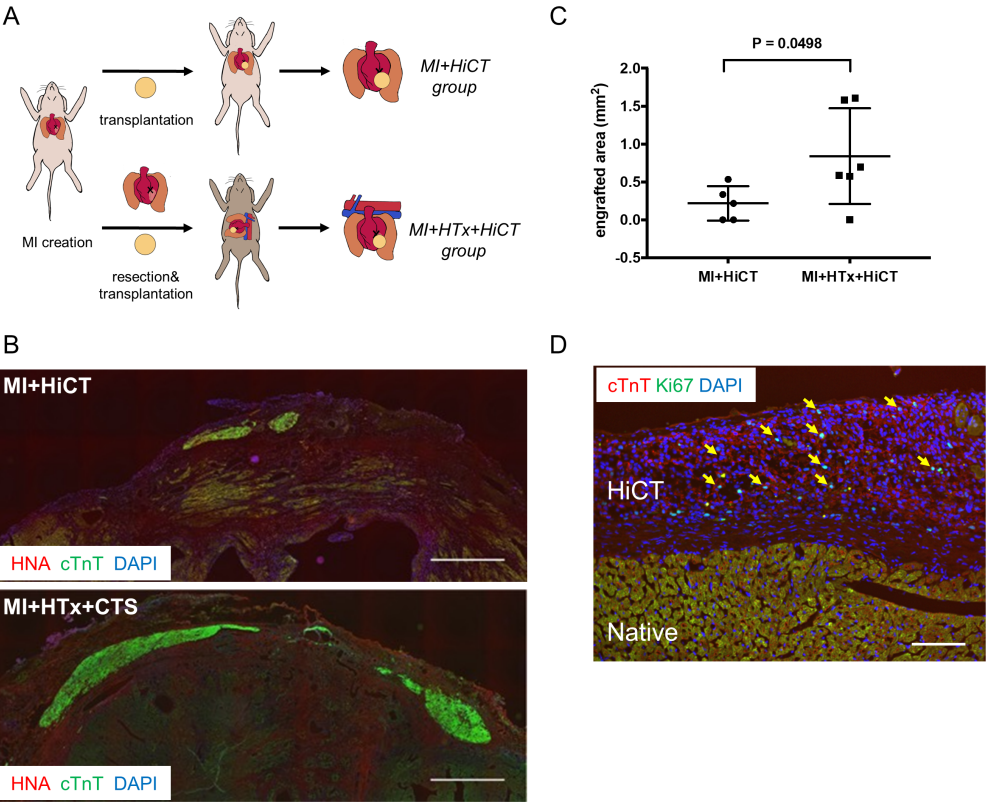
Figure3



709 **Figure4**



718 **Figure5**



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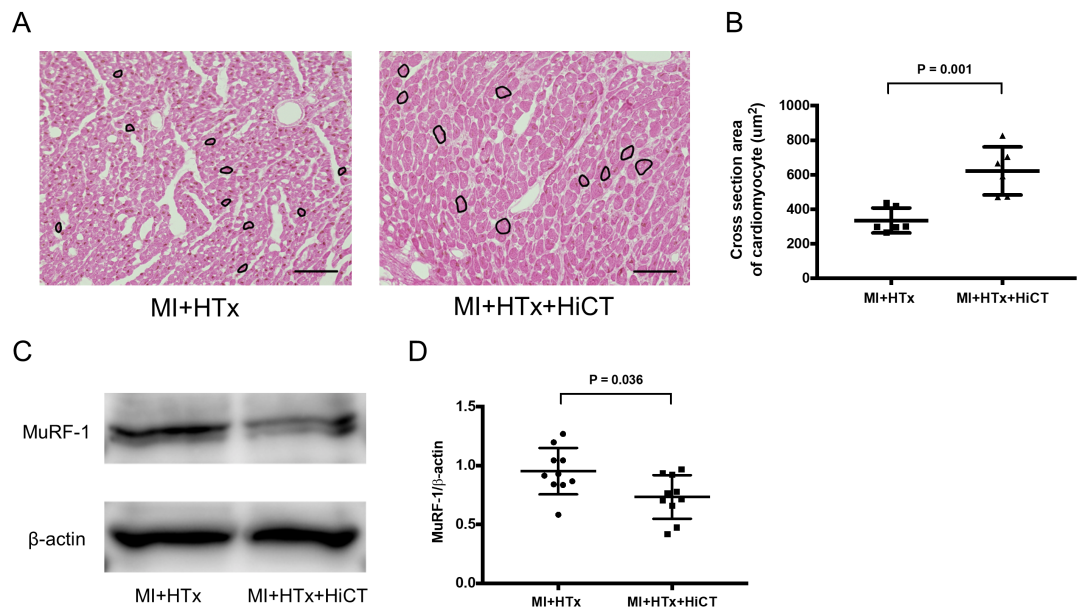
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727 **Figure6**



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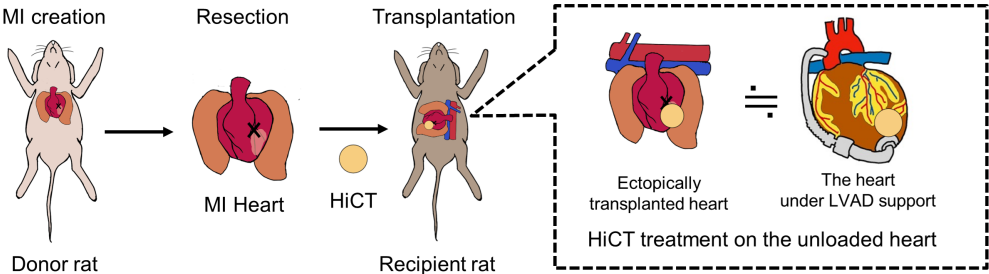
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736 **Figure7**



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