Functional Consequences of Human Induced Pluripotent Stem Cells Therapy: Myocardial ATP Turnover Rate in the in vivo Swine Hearts with Post-Infarction Remodeling

Running title: Xiong et al.; hiPSC-derived vascular cells

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Abstract:

**Background**—The use of cells derived from hiPSCs as cellular therapy for myocardial injury has yet to be examined in a large-animal model.

**Methods and Results**—Immuno-suppressed, Yorkshire pigs were assigned to one of three groups; MI: distal LAD ligation and reperfusion (n=13), CELL: MI with 4x10⁶ hiPSC-VCs administered via a fibrin patch (n=14), Normal (n=15). At 4 weeks, LV structural and functional abnormalities were less pronounced in CELL hearts than in MI hearts (p<0.05), and these improvements were accompanied by declines in scar size (10.4±1.6% vs. 8.3±1.1%; MI vs CELL, p<0.05). CELL was associated with significant increase of vascular density and blood flow (0.83±0.11 and 1.05±0.13 ml/min per g, MI vs. CELL, p<0.05) in the peri-scar border zone (BZ), which was accompanied by improvements in systolic thickening fractions (IZ: -10±7% vs. 5±5%; BZ: 7±4% vs. 23±6%; p<0.05). hiPSC-VC transplantation stimulated c-kit⁺ cell recruitment to BZ and the rate of bromodeoxyuridine incorporation in both c-kit⁺ cells and cardiomyocytes (p<0.05). Using a magnetic resonance spectroscopic saturation transfer technique, we found that the rate of ATP hydrolysis in BZ of MI hearts was severely reduced, the severity of this reduction was linearly related to the severity of the elevations of wall stresses (r=0.82, p<0.05). This decline in BZ ATP utilization was markedly attenuated in the CELL group.

**Conclusions**—hiPSC-VC transplantation mobilized endogenous progenitor cells into the BZ, attenuated regional wall stress, stimulated neovascularization, and improved BZ perfusion, which in turn resulted in marked increases of BZ contractile function and ATP turnover rate.

**Key words:** heart, adenosine–triphosphate, stem cell, myocardium, hypertrophy
Introduction

Heart failure is the end-stage clinical syndrome for a variety of cardiovascular diseases and affects over 23 million patients worldwide, including 5.8 million in the US.\(^1,2\) The healthcare expenses associated with heart failure cost the US government more than $39 billion annually.\(^1,2\) Heart failure often develops after acute myocardial infarction (MI), because the injured myocardial tissue fails to recover or regenerate.\(^3\) The heart has long been considered a post-mitotic organ and incapable of self-renewal; however, recent reports have demonstrated that progenitor cells mediate cardiomyocyte turnover in normal adult hearts,\(^4\) and that heart tissue can regenerate after injury through the differentiation of cardiac progenitor cells (CPCs) and through activation of the cell cycle in cardiomyocytes.\(^5\) The endogenous rate of cardiomyocyte regeneration is too slow to replace the cardiomyocytes that are lost during ischemia, but these mechanisms of cardiomyocyte turnover may be a key component of the therapeutic effects associated with cellular therapies,\(^6-8\) which have recently been shown to benefit patients with postinfarction LV remodeling.\(^9,10\)

Perhaps one of the most significant recent achievements in medical science has been the development of induced pluripotent stem cells (iPSC).\(^11,12\) These cells reproduce much of the regenerative potentials possessed by pluripotent embryonic stem cells, but are generated from a patient’s own somatic cells and, consequently, minimize the ethical concerns and potential immunogenic complications associated with embryonic stem cell therapies. The myocytes generated through cellular therapy, or from the activity of endogenous progenitor cells, are believed to reduce left ventricular (LV) dilatation and bulging at the site of infarction, which subsequently decreases myocardial wall stress and improves myocardial function and metabolism;\(^7,13,14\) however, the use of iPSCs or cells derived from iPSCs for the treatment of
ischemic myocardial injury has not yet been examined in a clinically relevant large-animal model.

The hypothesis that cardiac dysfunction in failing hearts develops because of energy starvation is an old one; however, whether the decreased reserve of myocardial ATP turnover rate contributes to the progression of cardiac dysfunction is largely a topic for speculation as the accurate measurements of myocardial ATP turnover rates cannot be examined in vivo. Although magnetic resonance spectroscopy-magnetization saturation transfer (MRS-MST) technique has been extensively used to measure the ATP flux via creatine kinase (CK), the accurate evaluation of ATP turnover rates via mitochondrial and cytosolic enzymes (ADP+Pi→ATP) has generally not been successful in vivo because the level of myocardial free inorganic phosphate (Pi) is too low to be measured directly.

Here, we introduce a novel MRS-MST method enabling us to calculate the ATP hydrolysis rate without measuring Pi levels (see Online supplemental for mathematical demonstration), which overcomes the primary barrier in determining the ATP turnover rate in vivo. The accuracy of this method was rigorously examined and confirmed in both skeletal muscle and the heart under baseline and high cardiac workloads. Using an immuno-suppressed swine model of postinfarction LV remodeling and this novel NMR technique, we examined the hypothesis that in the in vivo hearts with postinfarction LV remodeling, the reserve of myocardial ATP turnover rate is decreased. We hypothesized that treatment with an epicardial fibrin patch enhanced delivery of vascular cells derived from human iPSCs (hiPSC-VCs) would decrease the peri-scar border zone myocardium (BZ) overstretch and wall stresses, which in turn, result in an improvement in myocardial ATP turnover rate.
Methods

A more detailed description of the experimental procedures used in this investigation is provided in the Supplemental Materials.

Generation of vascular cells (VC) from human induced pluripotent stem cells (hiPSC)

The characteristics of the hiPSCs used for these studies were provided in: “Clinical Scale Derivation of Natural Killer Cells from Human Pluripotent Stem Cells for Cancer Therapy, David A. Knorr et al. Stem Cells Translational Medicine, in press”. The generation of iPSCs from neonatal human dermal fibroblasts (NHDFs) was accomplished by lentiviral transduction of OCT4, SOX2, KLF4, and cMYC.18 The iPSC criteria include PCR and immunostaining of pluripotent markers including Oct3/4, Sox2, Nanog, SSEA-4, and Tra-1-81, ability to form teratomas and normal karyotype. hiPSCs expressing GFP were separately generated using Sleeping Beauty transduction method (Amaxa, Gaithersburg, MD), as previously described.19 The GFP+ hiPSCs were then subject to vascular differentiation using a previously published protocol for human embryonic stem cells.20 The vascular cells derived from hiPSC (hiPSC-VC) consisted of two distinct cell types, corresponding to endothelial (EC) and smooth muscle cells (SMC).

Swine model of ischemia reperfusion and cell transplantation

The experimental protocol was approved by the University of Minnesota Research Animal Resources Committee. All experimental and animal maintenance procedures were performed in accordance with the Animal Use Guidelines of the University of Minnesota and were consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication No 85-23).

Female Yorkshire farm swine (~15 kg, Manthei Hog Farm, Elk River, MN) were
randomly assigned to one of three experimental groups: CELL, MI, and Normal. Ischemia-reperfusion injury was surgically induced in animals from the CELL and MI groups by temporarily ligating the coronary artery for 60 min as described previously and as summarized in the Supplemental Methods; animals in the Normal group underwent the same surgical procedure except for the ligation step. A fibrin patch containing 4 million hiPSC-VCs (hiPSC-ECs and hiPSC-SMCs, 2 million each) was placed over the ligation site of hearts in the CELL group. Because the transplanted cells were of human origin, the immune systems of animals in all three experimental groups were suppressed via cyclosporine injection (15 mg/kg per day, supplemented with food).7

Contractile function and infarct size

Cardiac functional parameters (LVEF and systolic thickening fraction) and infarct size were evaluated via MRI as summarized in the Supplemental Methods. Measurements were performed with a 1.5 Tesla clinical scanner (Siemens Sontata, Siemens Medical Systems, Islen NJ); functional parameters were determined from short-axis cine images, and infarct sizes were determined via delayed-enhancement MRI.

Myocardial ATP turnover rate

In vivo measurements of the myocardial ATP turnover rate were obtained via an open-chest 31P MRS-MST protocol as summarized in the Supplemental Methods. Measurements were performed on a 65 cm-bore 9.4-T magnet interfaced with a Vnmrj console (Varian, CA). Radiofrequency transmission and magnetic resonance spectroscopy (MRS) signal detection were performed with a double-tuned (1H and 31P) surface coil (28-mm diameter). Border zone measurements were obtained by suturing the coil directly to the LV epicardium over the peri-infarct region in hearts from the MI and CELL groups, and remote-zone measurements were
obtained by suturing the coil over a non-infarcted region; measurements in Normal hearts were obtained with the coil positioned on the anterior wall. Measurements were performed both under baseline condition and high cardiac workstate induced by catecholamine stimulation (dobutamine and dopamine, each 10 µg/kg/min iv).

A complete theoretical derivation of our experimental approach is provided in the Supplemental Materials. Briefly, the in vivo metabolism of ATP can be modeled as a chemical exchange network comprising two reactions among three components: the shuttling of inorganic phosphate (Pi) between phosphocreatine (PCr) and ATP (PCr↔ATP), and the hydrolysis/condensation of ATP (ATP↔ADP+Pi). The kinetics of ATP metabolism can be summarized with two pseudo-first-order rate constants: $k_{\text{PCr} \rightarrow \text{ATP}}$ for the forward creatine kinase reaction, and $k_{\text{ATP} \rightarrow \text{Pi}}$ for ATP→Pi hydrolysis reactions. The $k_{\text{ATP} \rightarrow \text{Pi}}$ includes contributions from: i) all of contraction/relaxation associated reactions that are energized by ATP, and ii) additional substantial contributions from a cytosolic near-equilibrium enzyme complex comprised of glyceraldehyde phosphate dehydrogenase and phosphoglycerate kinase (GAPDH/PDK). Because the PCr↔ATP reaction is at equilibrium, the rate of the ATP→ADP+Pi reaction can be calculated by subtracting the flux of the ATP→PCr reaction from the total ATP turnover rate (ATP→PCr plus ATP→ADP+Pi):

$$k_{\text{ATP} \rightarrow \text{Pi}} = \frac{M_{0,\text{ATP}} - M_{\text{ss,ATP}}}{M_{\text{ss,ATP}}} / T_{1,\text{ATP}} \cdot \frac{M_{0,\text{PCr}} - M_{\text{ss,PCr}}}{M_{\text{ss,PCr}}} / T_{1,\text{PCr}} \tag{1}$$

where $k_{\text{ATP} \rightarrow \text{Pi}}$ is the pseudo first-order rate constant of the ATP→ADP+Pi reaction; $M_0$ and $T_{1,\text{int}}$ are the fully relaxed magnetization and intrinsic longitudinal relaxation time constants of spin, respectively, as determined via $^{31}$P MR spectroscopy; $M_{\text{ss,ATP}}$ is the steady-state magnetization of ATP when PCr and Pi are completely saturated; and $M_{\text{ss,PCr}}$ is the steady-state magnetization of PCr when ATP is completely saturated. The measurement of $k_{\text{ATP} \rightarrow \text{Pi}}$ can be achieved from as
few as three spectra, one taken with the ATP\textsubscript{γ} signal saturated to measure the steady state magnetization of PCr (M\textsubscript{ss,PCr}), one taken with both Pi and PCr saturated to measure the steady state magnetization of ATP\textsubscript{γ} (M\textsubscript{ss,ATP\textsubscript{γ}}), and one taken with no saturation to measure the magnetization of PCr and ATP\textsubscript{γ}, (M\textsubscript{0,PCr} and M\textsubscript{0,ATP\textsubscript{γ}}). The validity of the methodology was rigorously examined on swine skeletal muscle (n=5) where Pi is reliably measureable; the results from the novel approach were compared to that from conventional MST method. Details of validation experiments are provided in Supplemental Materials.

**Myocardial perfusion, left-ventricular hemodynamics, and histological assessments**

Evaluations were performed after the chest had been opened for the \textsuperscript{31}P MRS assessments. Myocardial perfusion was measured via the injection of fluorescently labeled microspheres as described previously\textsuperscript{22-24}. LV hemodynamics was monitored via a catheter inserted through the apical dimple and into the LV\textsuperscript{22} and histological assessments were performed according to standard protocols. More detailed descriptions of these experimental procedures are provided in the Supplemental Methods.

**Statistics and data analysis**

All data are reported as mean ± SD. Statistical analyses were performed with Sigmastat version 3.5 (San Jose, Ca). Comparisons among groups were analyzed for significance with the one-way analysis of variance. Comparisons among different time points and between experimental groups were performed with two-way repeated measures analysis of variance. A value of p<0.05 was considered significant. When analysis of variance demonstrated a significant effect, post hoc analysis was performed using the t-test with Bonferroni correction. Comparisons of experimental condition versus baseline within a group were performed with a paired t-test. Comparisons between groups were performed with an unpaired t-test. Linear regression and correlation
analysis were performed to analyze the variables of rate-pressure product, LV systolic thickening fraction, end diastolic volume and wall stress, versus the myocardial flux rate of ATP hydrolysis reaction.

Results

Human induced pluripotent stem cell-derived vascular cells (hiPSC-VCs)

The hiPSC-VCs consisted of two distinct cell types: hiPSC-derived endothelial cells (ECs) and hiPSC-derived smooth muscle cells (SMCs) (Figure 1). The hiPSC-ECs expressed endothelial markers such as CD31, CD73, CD105, CD144, CD146 and vWF and formed robust tube-like structures when cultured in Matrigel (Figure 1 A-C). The hiPSC-SMCs displayed a typical smooth-muscle cell phenotype and expressed the smooth-muscle cell markers SMA, SM22, and calponin (Figure 1D). These results are consistent with those obtained when human embryonic stem cells were cultured with the same vascular differentiation protocol.\(^2\) hiPSC-VC transplantation reduced LV remodeling and improved cardiac performance after MI

One week after MI injury, infarcts were ~10% of the size of the left ventricle (LV) in both MI and CELL hearts (Figure 2A) and were accompanied by evidence of cardiac remodeling, such as cardiac hypertrophy (Figure 2B) and dilatation (Figure 2C); however, infarcts at Week 4 were significantly smaller, and remodeling was significantly less extensive, in CELL hearts than in MI hearts. MI injury also led to a significant decline in LV ejection fraction (LVEF) (Figure 2D). However, the systolic thickening fraction at the border zone of the infarct (Figure 2E) was significantly greater in CELL hearts than in MI hearts, and the bulging of the infarct was also prevented by CELL treatment (Figure 2E); these improvements were accompanied by declines
in measurements of myocardial wall stress at both the border zone and infarct zone (Figure 2F).

Cardiac hemodynamic parameters in MI and CELL hearts were similar to measurements in Normal hearts both at baseline and during high cardiac workstates (Table 1), which suggests that the injured hearts were in the compensated phase of LV remodeling. Typical cardiac MRI images of heart from each of the experimental groups at end diastole and end systole are shown in Figure 2G. Videos of typical heart from each of the experimental groups are illustrated in the Online Supplemental Materials.

**hiPSC-VC transplantation improved myocardial energetics after MI**

Recently, we used a novel $^{31}$P MRS two dimensional chemical shift imaging protocol to demonstrate that the effect of MI injury on myocardial biogenergetics is heterogeneous: PCr/ATP ratios declined near the border of the infarcted region, but not (or to a much lesser extent) in remote (i.e., non-infarcted) tissue. The results obtained in our current investigation with hiPSC-VCs are consistent with our previous report: the PCr/ATP ratio was significantly lower at the border-zone of the infarct in MI hearts than in Normal hearts under baseline workload conditions and significantly improved by hiPSC-VC treatment (Table 2). For the study reported here, we have further developed another $^{31}$P MRS protocol that enables us to measure, in vivo, the myocardial ATP turnover rate in different regions of the heart.

In the heart, ATP turnover can be modeled as a chemical exchange network with three components: PCr$\leftrightarrow$ATP$\leftrightarrow$Pi, and the kinetics of ATP metabolism can be summarized with two pseudo-first-order rate constants: $k_{\text{PCr}\rightarrow\text{ATP}}$ for the creatine kinase reaction and $k_{\text{ATP}\rightarrow\text{Pi}}$ for the ATP$\rightarrow$Pi reaction. The corresponding chemical reaction flux can be calculated by multiplying the rate constant with metabolite concentration: $\text{Flux}_\text{PCr}\rightarrow\text{ATP} = k_{\text{PCr}\rightarrow\text{ATP}} \times \text{[PCr]}$ and $\text{Flux}_\text{ATP}\rightarrow\text{Pi} = k_{\text{ATP}\rightarrow\text{Pi}} \times \text{[ATP]}$. Myocardial ATP turnover rates were determined in vivo as illustrated in
Figure 3, and were rigorously validated with equivalent in-vivo analyses performed on skeletal muscle (n=5, Supplemental Figure 1). The intrinsic T1 (T1\textsuperscript{int}) of ATP\textgamma and PCr were measured under baseline conditions via progressive saturation (Figure 3A, saturation of ATP\textgamma) and inversion recovery (Figure 3B, double saturation of both PCr and Pi), respectively. At 9.4 Tesla, T1\textsuperscript{int} in the heart was 1.1±0.1 sec for ATP\textgamma and 3.2±0.1 sec for PCr, which are in good agreement with measurements in skeletal muscle (Supplemental Table 1) and with previously reported values at the same magnetic field.\(^{25}\) These observations indicate that T1\textsuperscript{int} is determined by the microenvironment of the magnetic field surrounding a given molecule and is not affected by physiological or pathological conditions or by the kinetics of the chemical exchange network.

Kinetic measurements (k\textsubscript{PCr→ATP} and k\textsubscript{ATP→Pi}) were performed via a three-spectrum MST experiment (Figure 3C). In Normal hearts, Flux\textsubscript{ATP→Pi} measurements were linearly related to the rate-pressure product (RPP, LV pressure X heart rate) (Figure 3D), indicating that the ATP→Pi turnover rate and cardiac workload are tightly coupled. MI injury was associated with significantly lower k\textsubscript{ATP→Pi} measurements at the border zone of the infarct under both baseline and high-workload conditions (Figure 3E). However, k\textsubscript{ATP→Pi} measurements in the remote zone were unchanged (k\textsubscript{ATP→Pi} from remote zone of MI hearts (n=5) was 0.15±0.03 s\textsuperscript{-1}, p=NS vs. Normal hearts), indicating that the effect of infarction on ATP turnover rate is localized to the border zone at this phase of the LV remodeling. Although the RPP in response to catecholamine stimulation in MI and Normal hearts was similar (Table 1), the k\textsubscript{ATP→Pi} of BZ from MI hearts were less responsive. The decline in k\textsubscript{ATP→Pi} measurements observed at the border zone of MI hearts was significantly improved by hiPSC-VC treatment (Figure 3E). The border-zone Flux\textsubscript{PCr→ATP} was significant lower in MI hearts than Normal hearts. The k\textsubscript{PCr→ATP} measurements in Normal, MI, and CELL hearts were similar under both baseline and high-workload conditions.
Myocardial flux of the PCr–ATP and ATP–Pi reactions were summarized in Figure 4A. The concentration of ATP in the myocardium of swine hearts (5.3 μmol/gram wet wt) has been determined previously.26,27 Flux\textsubscript{PCr–ATP} and Flux\textsubscript{ATP–Pi} were significantly lower in the border-zone of both MI hearts and CELL hearts than in the corresponding region of Normal hearts, and Flux\textsubscript{ATP–Pi}, but not Flux\textsubscript{PCr–ATP}, was improved by hiPSC-VC treatment (Figure 4A). Flux\textsubscript{ATP–Pi} was also linearly correlated with measurements of the myocardial systolic thickening fraction, border-zone wall stress, and LV end-diastolic volume (Figure 4B-D). These findings are consistent with existing understanding that regional wall stress is a key determinant of metabolic activity and demand and contractile element response.28

Survival and engraftment of hiPSC-VCs in infarcted swine heart

The transplanted hiPSC-VCs had been engineered to express GFP; thus, the engraftment of hiPSC-VCs into the myocardial vasculature was evaluated by staining for the expression of GFP, CD31, and SMA, and proliferation was evaluated by staining for bromodeoxyuridine (BrdU) incorporation in the hearts of animals that had been infused with BrdU (10 mg/kg per day, administered via an insulin pump) for seven days. GFP\textsuperscript{+} cell counts indicated that the engraftment rate was 7.1±0.8% of the total number of cells transplanted per animal at Week 1 and 2.3±0.9% at Week 4 (Figure 5A). The transplanted hiPSC-VCs appeared to integrate into pre-existing vessels (Figure 5B) and to generate new vessel growth in the area of the fibrin patch (Figure 5C), and abundant evidence of BrdU incorporation by GFP\textsuperscript{+} cells confirmed that the engrafted hiPSC-VCs were highly proliferative (Supplemental Figure 2).

hiPSC-VC transplantation improved myocardial vascularity and perfusion after MI

MI injury led to a severe loss of vascularity, as evidenced by the minimal amount of CD31 and
SMA expression observed at the border zone of the infarct in hearts from the MI group; however, both CD31+ and SMA+ vascular density was significantly greater in CELL hearts than in MI hearts (Figure 5D-G). To determine whether the increase in vascular density was accompanied by a corresponding increase in blood flow, regional myocardial blood flow was measured via cMRI and microspheres injection29, 30 (n=4 per group), and coronary reserve was examined using adenosine-induced myocardial hyperemia (0.5 mg/kg per min, iv).22, 31 These data demonstrate that CELL treatment led to significant increases in BZ myocardial perfusion (Figure 5H), which were accompanied by significant improvements in systolic thickening fractions and ATP turnover rate in this region of the myocardium (Figure 4). Myocardial blood flow also tended to be higher in hearts from the CELL group during hyperemia (Figure 5I), but the difference between CELL and MI hearts was not significant (Figure 5I), and calculation of the coronary flow reserve did not identify significant differences among experimental groups or between different regions of myocardium (Figure 5I). Collectively, these observations demonstrate that the transplanted hiPSC-VCs were able to integrate into the growing vasculature and to generate new vessels suggesting the vasculogenesis. However, fewer than 5% of vessels expressed GFP, so the enhanced vascularity and perfusion associated with cell treatment likely evolved primarily through the sprouting of preexisting vessels.

**hiPSC-VC transplantation stimulated the activation and proliferation of endogenous CPCs and cardiomyocytes**

To determine whether cell therapy enhanced the activation and proliferation of endogenous CPCs, sections were stained for the presence of alpha-sarcomeric actin (to identify cardiomyocytes), for expression of the progenitor-cell marker c-kit, and for BrdU incorporation (Figure 6A-B, Supplemental Figure 3). Hearts from animals in the Normal group displayed
little evidence of c-kit expression, but c-kit+ cells were common in the injury site of hearts from the MI group, and the proportion of c-kit+ cells that were also positive for BrdU incorporation was significantly higher in MI hearts than in Normal hearts (Figure 6C-D). c-kit+ cells often co-expressed the cardiac-cell marker GATA-4, but cells that co-expressed c-kit and the hematopoietic-cell marker CD45 were rare (Figure 6E-F), which suggests that many of the progenitor cells were committed to the cardiac lineage, and that inflammatory cells were not a significant source of c-kit expression. Furthermore, both the number of c-kit+ cells and the incorporation rate of BrdU by c-kit+ cells were significantly greater in CELL hearts than in MI hearts at Week 1, but not at Week 4. Thus, the transplanted hiPSC-VCs appeared to enhance the endogenous response to myocardial infarction by transiently increasing both the number and the proliferation of CPCs in the infarcted region. The proportion of cardiomyocytes that were positive for BrdU incorporation was also significantly higher in the border zone of hearts from the CELL group than in the corresponding region of hearts from MI animals (Figures 7A-E) and BrdU+ cardiomyocytes were significantly smaller than BrdU− cardiomyocytes from the same region (Figure 7F), which suggests that the enhanced activation of CPCs was accompanied by increases in cardiomyocyte turnover.

Discussion

One of the most unsettled questions in cardiovascular physiology is how the rate of ATP production and utilization is regulated in the heart and how the limitation of this rate may contribute to contractile dysfunction in hearts that become hypertrophic in response to MI-induced LV remodeling, pressure overload, or volume overload. The heart has the highest ATP turnover rate per gram of tissue of any organ in the body;32 however, the ATP reserve in
myocardial tissue is so low that it would be exhausted after only a few dozen beats if the ATP production machinery stopped functioning. In vivo methods for accurately measuring the ATP utilization rate in the heart have not been established, because multiple mechanisms for ATP turnover happen simultaneously, and the conventional method requires quantification of the intracellular Pi level, which is intrinsically low, and overlaps with 2,3-diphosphoglycerate (2,3-DPG) resonance peaks. In addition, whether the intracellular levels of myocardial free Pi are completely NMR visible is also somewhat controversial. In this report, we introduce a novel, double-saturation $^3$P MST protocol that measures the ATP utilization rate without measuring Pi, thereby circumventing the barrier for in vivo applications (Supplemental Materials). By applying this NMR method, we have observed abnormalities in the rate of ATP utilization at the BZ of infarction; furthermore, the BZ bioenergetic abnormality was significantly corrected in response to CELL therapy, and this improvement was associated with significantly increased BZ contractile function (Figures 2 and 4). The observation that ATP hydrolysis/synthesis rates increased in the BZ of CELL treated hearts (Figure 4) is not surprising, given the significant volume of literature suggesting that the factors responsible for myocyte overstretch and elevations in wall stress also lead to a decline in contractile function, and that correcting these factors would contribute to increases in the BZ systolic thickening fraction. However, in the in-vivo heart, the mechanisms responsible for this simple observation likely involve multiple factors throughout the network of ATP machinery, which functions seamlessly to maintain the balance between energy delivery and demand for the contractile apparatus. A more comprehensive and inclusive discussion of these mechanisms is beyond the scope of the present investigation, but future studies combining measurements of in-vivo ATP turnover rates and intermediary metabolism, with measurements of changes in protein activity, the expression
levels of CK isozymes and ATPase subunits, and isolated mitochondrial function could advance our understandings of the molecular pathways that are activated by myocyte hypertrophy, over-stretching, and regional LV wall stress.

Validation of ATP utilization rate measurements in skeletal muscle

Methods for accurately measuring the total rates of myocardial ATP synthesis (or utilization) in the in vivo heart have not been established because of difficulties in accurate measurement of myocardial free Pi level. In this report, we have introduced and rigorously validated novel 31P MST protocol that measures the ATP utilization rate without the need for a Pi measurement. The validation experiments were performed in swine skeletal muscle, in which adequate Pi resonance definition ensured that measurements could be made with both the new and the classical MST techniques (Supplemental Figure 1). The equivalence of the results obtained with the novel and classic methods validates our new method for measuring the kATP→Pi and FluxATP→Pi in the in vivo heart where myocardial free Pi level is too low to be accurately quantified.

Myocardial ATP turnover rate in in vivo heart

In response to acute MI, molecular, structural, and functional abnormalities first occur in the border zone. The surviving BZ myocytes are over-stretched, and then spread to adjacent regions of healthy myocardium until the entire LV is affected and signs of heart failure appear. Our findings demonstrate that the FluxATP→Pi rate in MI hearts is highly heterogeneous, with a pronounced reduction in the border zone but not in the remote zone. Although the underlying mechanisms responsible for the decline in border-zone FluxATP→Pi and its failure to increase in response to catecholamine stimulation are beyond the scope of this investigation, these mechanisms are likely similar to those present in myocardium of failing hearts, which show down regulation to catecholamine stimulation.
The decline in border-zone \( \text{Flux}_{\text{ATP} \rightarrow \text{Pi}} \) was accompanied by contractile dysfunction, a loss of vascular density, and elevated wall stress. Previous reports using both a localized \(^{31}\)P MRS protocol and more recently a two-dimensional chemical shift imaging technique\(^7,27\) have shown that the \( \text{PCr/ATP} \) ratio and, by extension, the bioenergetic efficiency of the border zone is impaired. This abnormal bioenergetic status is related to elevations in border-zone wall stress, which could contribute to the progression of the disease state from compensated MI to congestive heart failure. The decline in border-zone \( \text{Flux}_{\text{ATP} \rightarrow \text{Pi}} \) is also consistent with previous observations that the number of \( F_1F_0\)-ATPase subunits is severely reduced in the border zones of compensated swine hearts with LV remodeling.\(^{27,36}\) Our observation that the ATP hydrolysis rate is severely reduced in border-zone myocardium that was recovered in response to the patch enhanced cell treatment suggest that cellular therapy targeting the border zone of hearts in the compensated phase of remodeling may prevent progression to heart failure (Figure 4 C-D).

The transplantation of hiPSC-VCs corrected the regional abnormalities in ATP hydrolysis rate that were observed in MI hearts (Figure 4). This beneficial effect on ATP hydrolysis supports the previous observation that the cell transplantation into infarcted hearts improves myocardial bioenergetics as indicated by the \( \text{PCr/ATP} \) ratio,\(^7,13,14\) and that these bioenergetic benefits were accompanied by significant improvements in border-zone myocardial contractile function, wall stress, vascular density, and myocardial blood flow. Future investigations are warranted to identify the molecular pathways that are induced by myocyte over-stretching and support the beneficial effects of cardiac cell therapy.

The hypothesis that failing hearts are energy starved is an old one.\(^{15}\) Based on the studies using the animal models of heart failure and patients with LVH, it is a rather consistent finding that myocardial HEP levels and forward flux rate of CK is significantly reduced, which is
linearly related to the severity of LV dysfunction.\textsuperscript{22, 26} Myocardial HEP levels\textsuperscript{22, 31} and CK kinetics have been extensively studied.\textsuperscript{26} However, the rates of ATP hydrolysis have been largely overlooked in in-vivo investigations due to the intrinsically low myocardial Pi level. In the in vivo heart, myocardial Pi produces a weak signal that overlaps with the resonance peaks of 2,3-DPG from erythrocytes in the LV chamber blood. Our novel double-saturation $^{31}$P MRS protocol enables the ATP hydrolysis rate to be calculated without quantification of the Pi signal, thereby providing a new approach for studying myocardial ATP turnover rate in vivo (Supplemental Materials). The ATP hydrolysis rate is important as it reflects the total ATP utilization rate combining the contractile apparatus utilization and all other ATPase pumps that maintain the structural integrity.

**Transplanted hiPSC-VCs enhanced the activation and proliferation of endogenous CPCs for cardiac repair**

The paradigm of the heart as a post-mitotic organ incapable of regeneration has been challenged by the discovery that cardiomyocytes can re-enter the cell cycle and begin dividing and by the presence of cardiac progenitor cells in the adult heart that are capable of generating new cardiomyocytes.\textsuperscript{4, 5, 7, 8, 39, 40} Here, we show that MI led to the activation of c-kit$^+$ cells at the injury site, which is consistent with previous reports.\textsuperscript{8, 41} The higher c-kit$^+$ cell density in CELL hearts was accompanied by a significantly higher rate of BrdU incorporation (Figure 6 C), suggesting that hiPSC-VC transplantation also increased c-kit$^+$ cell proliferation. Our data also show that hiPSC-VC transplantation increased the BrdU incorporation rate of border-zone cardiomyocytes at Week 4 after injury. Collectively, these observations suggest that hiPSC-VC transplantation activates endogenous progenitor cells, which may contribute to the increased myocyte turnover.
In conclusion, the present study introduces a novel MRS-MST approach for obtaining in-vivo measurements of the myocardial ATP hydrolysis rate. The ATP hydrolysis rate was linearly related to the RPP but was significantly reduced in the border zone of infarction, perhaps because of increases in wall stress in this region. Transplantation of hiPSC-VCs significantly increased the activation of endogenous CPCs in response to MI injury, which was accompanied by significant improvements in myocardial wall stress, vascular density, infarct size, and contractile function, and the ATP utilization rate. These beneficial effects may impede progression from the compensated phase of myocardial hypertrophy to heart failure.

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**Conflict of Interest Disclosures:** None.

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### Table 1. Hemodynamic data.

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<th>High Cardiac Workload</th>
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<tr>
<td>Normal (n=10)</td>
<td>92±11</td>
<td>98±7</td>
</tr>
<tr>
<td>MI (n=9)</td>
<td>96±12</td>
<td>94±5</td>
</tr>
<tr>
<td>CELL (n=9)</td>
<td>98±13</td>
<td>91±8</td>
</tr>
</tbody>
</table>

Data are Mean ± SD. *, p<0.05 vs. MI hearts. HR, heart rate; bpm, beats per min; LVSP, left ventricular systolic pressure; LVEDP, left ventricle end-diastolic pressure; RPP, rate-pressure product (HR X LVSP).

### Table 2. ³¹P MRS measurement data in *in vivo* heart

<table>
<thead>
<tr>
<th></th>
<th>Normal (n=10)</th>
<th>MI-BZ (n=9)</th>
<th>CELL-BZ (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>HWL</td>
<td>Baseline</td>
</tr>
<tr>
<td>PCr/ATP</td>
<td>2.09±0.09</td>
<td>1.94±0.23</td>
<td>1.64±0.22#</td>
</tr>
</tbody>
</table>

Data are Mean ± SD. #, p<0.05 vs. Normal hearts; *, p<0.05 vs. MI-BZ; ‡, p<0.05 vs. baseline for the same group. HWL, high cardiac workload; MI-BZ, border zone of MI hearts; CELL-BZ, border zone of CELL hearts.

### Figure Legends:

**Figure 1.** Characterization of human induced pluripotent stem cell-derived vascular cells (hiPSC-VCs). Panel A, flow cytometric analysis of hiPSC-derived endothelial cells (ECs), demonstrating the expression of CD31, CD73, CD105 and CD146. Blue line showed the samples...
stained with respective antibodies, with red line showing the isotype control. Panels B and D, representative immuno-staining images of hiPSC-ECs and smooth muscle cells (SMCs), respectively. The ECs expressed endothelial specific markers such as CD31, von Willebrand factor (vWF) and CD144 whereas the SMCs expressed smooth muscle actin (SMA), SM22 and Calponin. Panel C, hiPSC-ECs formed capillary tube like structure after 24 hr culture in matrigel.

**Figure 2.** Structural and functional benefits of hiPSC-VC transplantation. Panel A, At Week 4 post infarction, the CELL group showed significantly smaller infarct size (delayed enhancement MRI) as compared to MI group. Panel B, myocardial infarction induced left ventricular hypertrophy (LVH) as evidenced by significantly increased left ventricular weight over body weight (LVW/BW). The hiPSC-VCs transplantation attenuated the LVH. Panel C, myocardial infarction induced dilatation of LV chamber as compared to Normal group. The hiPSC-VC transplantation attenuated the chamber dilation. LVEDV: LV end-diastolic volume. Panel D, hiPSC-VCs transplantation significantly improved the ejection fraction of infarcted swine hearts. Panel E, the border zone (BZ) myocardium in post-infarcted swine hearts showed significant contractile dysfunction (systolic thickening fraction), which was significantly improved in response to hiPSC-VC transplantation. The hiPSC-VC treatment also prevented the infarct zone (IZ) bulging out during systole (negative thickening fraction). Panel F, hiPSC-VC transplantation alleviated the abnormal myocardial wall stress in the border zone and infarct zone myocardium. Panel G, representative cardiac MR images from NORMAL, MI and CELL groups at end-systole (left) and end-diastole (right). #, p<0.05 vs. Normal group; *, p<0.05 vs. MI group. Quantification of cardiac functional parameters was based on Normal hearts (n=10),
MI hearts (n=9), and CELL hearts (n=9).

**Figure 3.** Measurement of in vivo myocardial ATP turnover rate using $^{31}$P MRS-MST experiments. **Panels A and B,** measurement of intrinsic T$_1$ ($T_{1\text{int}}$) for PCr and ATP using progressive saturation (A) and inversion recovery (B) experiments. Spectra a1-a7 are progressive saturation experiment with ATP saturated (bold arrow) for 0.44, 0.88, 1.32, 2.2, 3.52, 5.28 and 7.04 sec, respectively. Spectra b1-b6 are inversion recovery experiment with both Pi and PCr saturated (bold arrows) for 0.44, 0.88, 1.32, 1.76, 2.64 and 3.96 sec, respectively. The magnetizations of PCr and ATP are quantified and then subject to exponential models to fit the intrinsic T$_1$s. The experiments yielded an average intrinsic T$_1$ of 3.2±0.1 sec and 1.1±0.1 sec for PCr and ATP, respectively. **Panel C,** three-spectrum MST experiment to measure the myocardial ATP turnover rate based on equation [1]. c1: control spectrum without saturation to quantify the $M_{0,\text{PCr}}$ and $M_{0,\text{ATP}}$; c2: ATP-saturated spectrum to quantify the $M_{ss,\text{PCr}}$; c3: Pi- and PCr-saturated spectrum to measure the $M_{ss,\text{ATP}}$. BISTRO-based frequency selective saturation pulses were indicated by bold arrows. Detailed information of BISTRO saturation pulse sequence is shown in Supplemental Figure 4. **Panel D,** scatter plot of Flux$_{\text{ATP}\rightarrow\text{Pi}}$ vs. rate-pressure product (RPP) for individual hearts from Normal group, indicating a tight coupling between the ATP hydrolysis rate and cardiac workload ($R^2$=0.89, p<0.05). **Panels E and F,** summary of myocardial $k_{\text{ATP}\rightarrow\text{Pi}}$ and $k_{\text{PCr}\rightarrow\text{ATP}}$ for all groups at both baseline and high cardiac work load (HWL) conditions. $k_{\text{ATP}\rightarrow\text{Pi}}$ is significantly reduced in BZ of MI hearts as compared to Normal hearts, whereas the $k_{\text{PCr}\rightarrow\text{ATP}}$ were similar among all groups and all conditions. In response to hiPSC-VC transplantation, the abnormality of ATP turnover rate in BZ was abolished. Flux$_{\text{ATP}\rightarrow\text{Pi}}$ was calculated from $k_{\text{ATP}\rightarrow\text{Pi}}$ *[ATP]. [ATP] value was estimated to be 5.3
µmol/gram wet tissue for normal myocardium. #, p<0.05 vs. Normal group; *, p<0.05 vs. MI group; ‡, p <0.05 vs. the same group at Baseline condition. Quantification of myocardial ATP turnover rate was based on Normal hearts (n=10), MI hearts (n=8), and CELL hearts (n=9).

Figure 4. Correlation of myocardial ATP turnover rate with severity of post-infarction LV remodeling. Panel A, energy transfer flux of ATP→PCr and ATP→Pi reactions from groups of Normal, MI and CELL hearts. #, p<0.05 vs. Normal; *, p<0.05 vs. MI. Panels B-D, relationship between myocardial FluxATP→Pi and BZ myocardium systolic thickening fraction, left ventricular end-diastolic volume (LVEDV) and BZ myocardial wall stress, respectively.

Figure 5. Functional engraftment and structural benefit of hiPSC-VC transplantation. Panel A, the engraftment rate of hiPSC-VCs at both Week 1 and Week 4 post transplantation. The calculation was based on GFP+ cell counts in histology analysis and an initial cell number of 4 million. Panel B, transplanted hiPSC-VCs contributed to neo-vascularization through engraftment onto vascular structures. Figure inset i-iv, co-staining of GFP (green) and smooth muscle actin (SMA, red) demonstrated hiPSC-VCs grafting onto a host artery (arrowhead). Panels C-F showed representative vascular staining from (C) a CELL heart at patch/myocardium border, (D) a Normal heart, (E) an MI heart at border zone, (F) a CELL heart at border zone. Vessels were visualized by CD31 staining (green) and smooth muscle actin staining (SMA, red), whereas myocardium was visualized by cardiac troponin I staining (cTnI, blue). Panel G summarized the quantification of arteriolar density (SMA+ vessels with diameter >25 µm) as well as total vascular density (all CD31+ vessels). Panels H and I summarized the microsphere-based myocardial blood flow (MBL) data examined at 4 weeks post infarction.
MBF measurements were performed at both baseline (H) and hyperemia (I, Adenosine, 0.5 mg/kg/min, iv) conditions. Myocardium samples (~1 gram) from either border zone (BZ) or remote zone (RZ) were examined. #, p<0.05 vs. Normal; *, p<0.05 vs. MI. All quantifications were performed based on data from 4 hearts each group.

**Figure 6.** Activation of c-kit⁺ progenitor cells in response to myocardial injury and hiPSC-VCs transplantation. **Panels A-B**, representative immuno-staining images from (A) Normal and (B) infarct area of CELL hearts at 1 week post surgery. Cardiac progenitor cells were identified by c-kit staining (green), cardiomyocytes were identified by alpha sacromeric actin staining (αSA, yellow), and cycling cells were identified by BrdU staining (red). Arrow in figure inset a1-a4 indicate c-kit⁺/BrdU⁻ cell whereas arrowheads in figure inset b1-b4 indicate c-kit⁺/BrdU⁺ cells. **Panels C and D**, quantification of BrdU incorporation rate of c-kit⁺ cells (BrdU⁺-c-kit⁺/c-kit⁺ %) and c-kit⁺ cell density, respectively. All quantification was performed based on 5 slides per heart and 3 heats per group. #, p<0.05 vs. Normal; *, p<0.05 vs. MI; ‡, p<0.05 vs. the same group at Week 1. **Panel E1-E4**, co-staining of CD45 and c-kit demonstrated that hematopoietic cells did not contribute significantly to the c-kit⁺ cells identified at 1 week post surgery. Arrow indicated one c-kit⁺/CD45⁺ cell (arrow). **Panel F1-F4**, co-staining of c-kit and GATA4 indicated the cardiac commitment of these progenitor cells. Arrows highlighted two GATA4⁺/c-kit⁺ cells.

**Figure 7.** hiPSC-VCs transplantation stimulated cardiac turnover. **Panels A-D**, cardiomyocyte turnover is evidenced by co-staining of alpha sarcomeric actin (αSA, green) and BrdU (red). Representative BrdU⁺ myocytes with higher magnification are shown in figure insets i-viii and denoted by arrowheads. BZ: border zone. RZ: remote zone. **Panel E**, quantification of myocardial regeneration level in terms of percentage of BrdU⁺ myocyte over total number of
myocyte (BrdU+ myocytes %). The quantification was performed based on 10 slides per location (BZ and RZ) per time point (Week 1 and Week 4), and 3 hearts per group (MI and CELL). An average of >10000 cardiomyocytes were analyzed per group. *, p<0.05 vs. RZ from the same time point within the same group; †, p<0.05 vs. MI group from the same region and same time point; ‡, p<0.05 vs. the same group at Week 1. Panel F, quantification of myocyte size (cross-section diameter) from BZ myocardium of CELL heart at Week 4. The BrdU+ myocytes were significantly smaller than BrdU- myocytes.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7

(A) MI-BZ 1WK, αSA, BrdU, DAPI

(B) CELL-BZ 1WK

(C) MI-BZ 4WK

(D) CELL-BZ 4WK

(E) BrdU+ Myocytes (%)

(F) Cross-section diameter (μm)

E: MI: ■ | CELL: □

F: p<0.05

BrdU+ CM | BrdU− CM
Functional Consequences of Human Induced Pluripotent Stem Cells Therapy: Myocardial ATP Turnover Rate in the in vivo Swine Hearts with Post-Infarction Remodeling
Qiang Xiong, Lei Ye, Pengyuan Zhang, Michael Lepley, Jinfeng Tian, Jun Li, Liying Zhang, Cory Swingen, J. Thomas Vaughan, Dan S. Kaufman and Jianyi Zhang

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SUPPLEMENTAL MATERIALS

SUPPLEMENTAL THEORY

Conventional magnetization saturation transfer (MST) experiment

The \textit{in vivo} metabolism of adenosine triphosphate (ATP) can be modeled as a chemical exchange network among phosphocreatine (PCr), ATP, and inorganic phosphate (Pi):

\[
\text{PCr} \xleftrightarrow{k_{\text{PCr}\rightarrow\text{ATP}}} \text{ATP} \xleftrightarrow{k_{\text{ATP}\rightarrow\text{PCr}}} \text{Pi} \quad [1]
\]

where the PCr$\leftrightarrow$ATP exchange is catalyzed by creatine kinase (CK), the ATP$\leftrightarrow$Pi exchange incorporates all other cellular activities that produce and consume ATP,\(^1\) and \(k\) is the pseudo-first-order rate constant for the unidirectional reactions.

Conventionally, the evolution of visible MR magnetizations during chemical exchange are modeled with the modified Block-McConnell equations:\(^2\), \(^3\)

\[
\frac{dM_{\text{PCr}}(t)}{dt} = \frac{M_{0,\text{PCr}} - M_{\text{PCr}}(t)}{T_{1,\text{PCr}}} - k_{\text{PCr}\rightarrow\text{ATP}}M_{\text{PCr}}(t) + k_{\text{ATP}\rightarrow\text{PCr}}M_{\text{ATP}}(t) \quad [2]
\]

\[
\frac{dM_{\text{Pi}}(t)}{dt} = \frac{M_{0,\text{Pi}} - M_{\text{Pi}}(t)}{T_{1,\text{Pi}}} - k_{\text{Pi}\rightarrow\text{ATP}}M_{\text{Pi}}(t) + k_{\text{ATP}\rightarrow\text{Pi}}M_{\text{ATP}}(t) \quad [3]
\]

\[
\frac{dM_{\text{ATP}}(t)}{dt} = \frac{M_{0,\text{ATP}} - M_{\text{ATP}}(t)}{T_{1,\text{ATP}}} + k_{\text{PCr}\rightarrow\text{ATP}}M_{\text{PCr}}(t) + k_{\text{Pi}\rightarrow\text{ATP}}M_{\text{Pi}}(t) - (k_{\text{ATP}\rightarrow\text{PCr}} + k_{\text{ATP}\rightarrow\text{Pi}})M_{\text{ATP}}(t) \quad [4]
\]

By selectively saturating the ATP\(\gamma\) magnetization during the MST experiment, equations \([2]-[4]\) change to:

\[
\frac{dM_{\text{PCr}}(t)}{dt} = \frac{M_{0,\text{PCr}} - M_{\text{PCr}}(t)}{T_{1,\text{PCr}}} - k_{\text{PCr}\rightarrow\text{ATP}}M_{\text{PCr}}(t) \quad [5]
\]

\[
\frac{dM_{\text{Pi}}(t)}{dt} = \frac{M_{0,\text{Pi}} - M_{\text{Pi}}(t)}{T_{1,\text{Pi}}} - k_{\text{Pi}\rightarrow\text{ATP}}M_{\text{Pi}}(t) \quad [6]
\]

\[
M_{\text{ATP}}(t) = 0 \quad [7]
\]

Equations \([5]\) and \([6]\) are mathematically equivalent and describe the unidirectional
kinetics of ATP production from PCr and Pi, respectively. Solving equations [5] and [6] yields:

\[ k_{\text{PCr(Pi)} \rightarrow \text{ATP}} = \left( \frac{M_{0,\text{PCr(Pi)}} - M_{\text{ss,PCr(Pi)}}}{M_{\text{ss,PCr(Pi)}}} \right) / T_{1,\text{PCr(Pi)}} \]  

where \( M_{\text{ss}} \) and \( M_0 \) represent the fully relaxed magnetizations with and without saturation of ATP\(_\gamma\), and \( T_{1,\text{int}} \) is the intrinsic longitudinal relaxation time constant. Thus, calculation of the ATP production rate constants via equation [8] requires two fully relaxed spectra: one control spectrum without saturation to obtain \( M_{0,\text{PCr(Pi)}} \) and one saturated spectrum with the saturation pulse set at the ATP\(_\gamma\) frequency to obtain \( M_{\text{ss,PCr(Pi)}} \). Intrinsic \( T_1 \) (\( T_{1,\text{int}} \)) is usually a constant among patients and unaffected by physiological or pathological conditions \(^4\)-\(^10\); however, if \( T_{1,\text{int}} \) is not known, it can be calculated from another mathematically equivalent equation:

\[ k_{\text{PCr(Pi)} \rightarrow \text{ATP}} = \left( \frac{M_{0,\text{PCr(Pi)}} - M_{\text{ss,PCr(Pi)}}}{M_{0,\text{PCr(Pi)}}} \right) / T_{1,\text{PCr(Pi)}} \]  

where \( T_{1,\text{app}} \) is measured directly via progressive saturation or inversion recovery experiments and then used to calculate \( T_{1,\text{int}} \) according to the following equation:

\[ (T_{1,\text{app}})^{-1} = (T_{1,\text{int}})^{-1} + k \]  

**Indirect \( ^{31} \text{P} \) MRS-MST method for measuring ATP turnover rates in vivo**

The conventional MST approach for measuring the ATP\( \leftrightarrow \)Pi rate constant in vivo relies on the quantification of Pi magnetization levels (\( M_{\text{Pi}} \)),\(^11\) which is difficult in vivo because myocardial Pi levels are intrinsically low, and because of overlap between the peaks for Pi and 2,3-diphosphoglycerate (2,3-DPG), which is generated from erythrocytes in the blood. Furthermore, there is some controversy about whether intracellular cardiac Pi is completely visible in an MR spectrum.\(^12\), \(^13\) Here, we introduce a novel \( ^{31} \text{P} \) MRS-MST approach that overcomes this difficulty by enabling us to measure the unidirectional ATP\( \rightarrow \)Pi rate constant indirectly, but without quantification of Pi.

The unidirectional reaction flux in chemical exchange network [1] can be expressed as:

\[ Flux_{\text{PCr} \rightarrow \text{ATP}} = k_{\text{PCr} \rightarrow \text{ATP}}[\text{PCr}] ; \quad Flux_{\text{ATP} \rightarrow \text{PCr}} = k_{\text{ATP} \rightarrow \text{PCr}}[\text{ATP}] \]  

[11]
Flux\(_{\text{Pi} \rightarrow \text{ATP}} = k_{\text{Pi} \rightarrow \text{ATP}}[\text{Pi}]\); \quad Flux_{\text{ATP} \rightarrow \text{Pi}} = k_{\text{ATP} \rightarrow \text{Pi}}[\text{ATP}] \quad [12]

Under homeostatic conditions, none of the metabolites (PCr, Pi, or ATP) accumulate or become depleted, indicating that the reversible reactions operate in equilibrium. Thus, the two unidirectional fluxes must be equal:\textsuperscript{1, 8, 10, 11}

\[ Flux_{\text{PCr} \rightarrow \text{ATP}} = Flux_{\text{ATP} \rightarrow \text{PCr}} \quad [13] \]

and the reverse rate constant for the ATP→PCr reaction could be expressed as:

\[ k_{\text{ATP} \rightarrow \text{PCr}} = \frac{[\text{PCr}]}{[\text{ATP}]} k_{\text{PCr} \rightarrow \text{ATP}} = \frac{M_{0,\text{PCr}}}{M_{0,\text{ATP}}} k_{\text{PCr} \rightarrow \text{ATP}} \quad [14] \]

where \(M_0\) represents the fully relaxed magnetization observed in an MR spectrum. By selectively saturating both the PCr and Pi magnetizations and observing the change in ATPγ magnetization, we can measure a new unidirectional rate constant (\(k_{\text{ATP},\text{tot}}\)) which represents the summation of both the ATP→Pi and ATP→PCr reactions.

\[ k_{\text{ATP},\text{tot}} = k_{\text{ATP} \rightarrow \text{PCr}} + k_{\text{ATP} \rightarrow \text{Pi}} \quad [15] \]

The measurement of \(k_{\text{r},\text{tot}}\) is performed via a method similar to the conventional MST approach. If both PCr and Pi magnetizations are saturated, the evolution of the ATPγ magnetization follows the equation:

\[
\frac{dM_{\text{ATP}_\gamma}(t)}{dt} = \frac{M_{0,\text{ATP}_\gamma} - M_{\text{ATP}_\gamma}(t)}{T_{1,\text{ATP}_\gamma}} - k_{\text{ATP},\text{tot}}M_{\text{ATP}_\gamma}(t) \quad [16]
\]

and solving equation [16] yields:

\[
k_{\text{ATP},\text{tot}} = \left(\frac{M_{0,\text{ATP}_\gamma} - M_{\text{ss,ATP}_\gamma}}{M_{\text{ss,ATP}_\gamma}}\right)^{-1}/T_{1,\text{ATP}_\gamma} \quad [17]
\]

where \(M_{\text{ss,ATP}_\gamma}\) and \(M_{0,\text{ATP}_\gamma}\) represent the fully relaxed magnetization of ATPγ with and without, respectively, saturation of both PCr and Pi. Combining equations [8], [14], [15] and [17] generates the following equation:

\[
k_{\text{ATP} \rightarrow \text{Pi}} = \left(\frac{M_{0,\text{ATP}_\gamma} - M_{\text{ss,ATP}_\gamma}}{M_{\text{ss,ATP}_\gamma}}\right)^{-1}/T_{1,\text{ATP}_\gamma} - \frac{M_{0,\text{PCr}}}{M_{0,\text{ATP}}} \left(\frac{M_{0,\text{PCr}} - M_{\text{ss,PCr}}}{M_{\text{ss,PCr}}}\right)^{-1}/T_{1,\text{PCr}} \quad [18]
\]

Equation [18] suggests that \(k_{\text{ATP} \rightarrow \text{Pi}}\) (the rate constant of ATP→Pi reaction) can be measured by subtracting \(k_{\text{ATP} \rightarrow \text{PCr}}\) (the rate constant of the reverse CK reaction) from \(k_{\text{ATP},\text{tot}}\) (the rate constant for the combined ATP→Pi and ATP→PCr reactions, measured
via a PCr-Pi double saturation experiment). Thus, once the chemical shift information of
Pi is known, this indirect approach enables both $k_{\text{PCr}\to\text{ATP}}$ and $k_{\text{ATP}\to\text{Pi}}$ to be calculated
from an MST experiment consisting of 3 spectra: 1) a control spectrum without
saturation to measure $M_{0,\text{PCr}}$ and $M_{0,\text{ATPγ}}$; 2) an ATPγ-saturated spectrum to measure
$M_{\text{ss,PCr}}$; and 3) a spectrum with both PCr and Pi saturated to measure $M_{\text{ss,ATPγ}}$. 
SUPPLEMENTAL METHODS

Generation of vascular cells from human induced pluripotent stem cells (hiPSC)
The generation of iPSCs from neonatal human dermal fibroblasts (NHDFs) was accomplished by lentiviral transduction of a polycistronic cassette harboring OCT4, SOX2, KLF4, and cMYC (pHAGE2-EF1α-STEMCCA, a gift from Dr. Gustavo Mostoslavsky, the Boston University School of Medicine). Three to six days post transduction, the cells were trypsinized and plated onto MEF cells in conditions to support iPSC growth. As early as day 7 of culture on MEFs, putative colonies with hiPSC morphology could be visualized. Colonies were allowed to expand for approximately 26 days before initial analysis. Individual colonies with hiPSC morphology from each sample were expanded and initially characterized by PCR-based gene expression and immunofluorescence of pluripotent markers. Suitable lines from matching typical iPSC criteria were selected for further studies. These iPSC criteria include immunofluorescence of pluripotent markers including Oct3/4, Sox2, Nanog, SSEA-4, and Tra-1-81, ability to form teratomas and normal karyotype. The characteristics of the hiPSCs utilized in the current study is provided in “Clinical Scale Derivation of Natural Killer Cells from Human Pluripotent Stem Cells for Cancer Therapy, David A. Knorr, et al. Stem Cells Translational Medicine, in press”. The hiPSC cell line was further engineered to constitutively express green fluorescence protein (GFP) using Sleeping Beauty transduction method (Amaxa, Gaithersburg, MD) as previously described. The methods of vascular differentiation of hiPSCs have been reported previously. Briefly, hiPSCs were maintained on mouse embryonic fibroblasts. In order to induce differentiation to vascular cell types, hiPSCs were co-cultured with M210 mouse stromal cells for 12-14 days in media containing 20% defined FBS, 1% L-Glutamine, 1% NEAA, and 0.1% β-mercaptoethanol. After this time, cells were magnetically sorted (EasySep, Stemcell Technologies, Vancouver, CA) for a CD34+CD31+ population. These cells were transferred to fibronectin-coated plates and grown in endothelial cell (EC) media, specifically EGM-2MV (Lonza, Basel, Switzerland) supplemented with rhVEGF (R&D Systems, Minneapolis, MN) to a concentration of 10 ng/mL. In order to generate the smooth muscle cell phenotype, the ECs were transferred to media containing platelet
derived growth factor-BB and transforming growth factor beta.

**Characterization of hiPSC-derived vascular cells (hiPSC-VCs)**

Detailed methods for characterizing the hiPSC-VCs via flow cytometry and immunostaining have been published previously. Briefly, flow cytometry analysis was performed with hiPSC-ECs that had been stained with fluorescently labeled (PE) antibodies against CD31, CD73, CD105, and CD146 or isotypes (BD Pharmingen, San Diego, CA, USA). For immunostaining of hiPSC-ECs, CD31 and CD144 were detected with mouse anti-human antibodies (eBioscience, San Diego, CA, USA and BD Pharmingen), and von Willebrand factor (vWF) was detected with mouse anti-human vWF (DAKO Carpinteria, CA, USA). Fluorescence was visualized with Cy3-conjugated donkey anti-mouse secondary antibodies (Jackson ImmunoResearch, PA, USA). The hiPSC-SMCs were examined for expression of α-smooth muscle actin (SMA), SM22, and calponin. SMA was detected using a mouse anti-α-SMC actin Cy3-conjugated antibody (Sigma). SM22 and calponin were detected with primary goat anti-human SM22 (Abcam, Cambridge, MA, USA) and mouse anti-human calponin (Sigma) antibodies, and then visualized with species-matched Cy3-labeled secondary antibodies. ProlongGold+DAPI (Invitrogen) was used for slide preparation and for visualizing nuclei via fluorescent microscopy. For the tube formation assay, 1X10⁵ hiPSC-ECs were seeded on top of matrigel and cultured for 24 hr.

**In vivo swine of ischemia reperfusion**

Female Yorkshire farm swine (~15 kg, Manthei hog farm, Elk River, MN) were randomly assigned to one of three experimental groups: CELL (n=14), MI (n=13), and Normal (n=15). The induction of experimental myocardial ischemia-reperfusion injury has been described previously. Briefly, animals were anesthetized with inhaled 2% isoflurane, intubated, and ventilated with a respirator and supplemental oxygen. Body temperature, ECG, blood pressure, and arterial oxygen saturation were monitored throughout the surgical procedure. A left thoracotomy was performed, and the root of the 1st and 2nd diagonal coronary arteries from the left anterior descending coronary artery (LAD) was occluded for 60 min and then reperfused, which typically leads to infarction in 10% of
the LV mass.\textsuperscript{19, 20} If ventricular fibrillation occurred, electrical defibrillation was performed immediately. Other drugs administrated during open-chest surgery included Lidocaine (2 mg/kg iv bolus before ligation followed by 0.05 mg/(kg·min) iv for 70 min) and Amoidorane (5 mg/kg iv bolus before ligation followed by 0.04 mg/(kg·min) iv for 70 min). The chest was then closed in layers and animals were allowed recover. Standard post-operative care, including analgesia, was administrated until animals ate normally and became active. Because the transplanted cells were derived from human cells, animals in all treatment groups received cyclosporine (15 mg/kg per day with food) for immunosuppression.

**Fibrin patch-based cell administration**

Immediately before transplantation, 2 million hiPSC-ECs and 2 million hiPSC-SMCs were harvested fresh from cell culture and re-suspended in 1 mL total volume of fibrinogen solution (25 mg/mL); then, the cell/fibrinogen solution was co-injected with a second solution containing catalytic thrombin (75 NIH units/mL), 4 mM CaCl$_2$ and 2 mM ε-aminocaproic acid onto the epicardium of the infarcted area. A plastic ring was placed on the heart to serve as a mold for the fibrin patch. The mixture usually solidified within 1 min, producing a circular fibrin patch of approximately 3 cm in diameter and 3 mm in thickness. A video showing the cell-containing final fibrin patch on top of a swine heart is available in the Online Data Supplement.

**Cardiac MRI**

MRIs were performed to assess cardiac function by using a 1.5 Tesla clinical scanner (Siemens Sontata, Siemens Medical Systems, Islen NJ) with a phased-array 4-channel surface coil and ECG gating\textsuperscript{21-23}. Animals were anesthetized with 2\% inhaled isoflurane and positioned in a supine position within the scanner. Cardiac function (ejection fraction and thickening fraction) was analyzed from short-axis cine images by using the QMASS program (Medis Medical Imaging Systems, Leiden, The Netherlands). Cine imaging was performed with the following MR parameters: TR=3.1 ms, TE=1.6 ms, flip angle=79°, matrix size=256x120, field of view=340x265 mm$^2$, slice thickness=6 mm (with a 4-mm gap between slices); 25 phases were acquired across the cardiac cycle.
Infarct size was measured via delayed enhancement MRI (0.20 mmol/kg gadopentetate dimeglumine, iv, bolus) and quantified with ImageJ software (National Institutes of Health). Delayed enhancement MRI was acquired with the following parameters: TR=16 ms, TE=4 ms, TI=300 ms, flip angle=30°, matrix size=256x148, field of view=320x185 mm², slice thickness=6 mm (with a 0-mm gap between slices).

Validation of the indirect ³¹P MRS approach in swine skeletal muscle
MRS-MST measurements were performed in skeletal muscle of the legs of swine (n=5) to validate the new approach. Briefly, animals were anesthetized with 2% isoflurane and ventilated with supplemental oxygen on a respirator. The skin of the rear thigh (3X3 cm²) was surgically removed and a double tuned (¹H and ³¹P) surface coil (28-mm diameter) was sutured onto the skeletal muscle. MR measurements were performed in a 65 cm-bore 9.4 Tesla magnet interfaced with a Vnmrj console (Varian, CA). ³¹P MR spectra were acquired with adiabatic half passage RF pulses for excitation to minimize flip angle variation due to B₁ inhomogeneity from the surface coil. Selective saturation of ATPγ or of both PCr and Pi was achieved by using the B₁-insentitive train to obliterate signal (BISTRO) technique as previously reported and illustrated in supplemental Figure 1. The complete MRS-MST protocol included the following 5 experiments/spectra: 1) Inversion recovery experiment with ATPγ saturated to measure the intrinsic T₁s of PCr and Pi; TR=12 sec. 2) Inversion recovery experiment with both PCr and Pi saturated to measure the intrinsic T₁ of ATPγ; TR=4 sec. 3) Fully relaxed control spectrum without saturation to measure M₀,PCr, M₀,ATPγ and M₀,Pi; TR=12 sec. 4) Spectrum with ATPγ saturated to measure Mₛₛ,PCr and Mₛₛ,Pi; TR=12 sec. 5) Spectrum with both PCr and Pi saturated to measure Mₛₛ,ATPγ; TR=6 sec. All spectra were acquired with 40 signal averages (NEX). The forward rate constants of PCr→ATP (k₉₉PCr→ATP) and Pi→ATP (k₉₉Pi→ATP) were determined via the conventional MST approach (by using equation [8] and the results from experiment/spectra 1, 3, and 4. The rate constants of k₉₉tot and k₉₉ATP→Pi were determined via our novel, indirect approach (by using equations [16] and [18] and the results from experiment/spectra 2, 3, and 5.

In vivo cardiac ³¹P MRS assessments
The surgical procedure for open-chest $^{31}$P MRS has been described previously. Briefly, animals were anesthetized with 2% isoflurane and ventilated with supplemental oxygen on a respirator. Polyvinyl chloride catheters (3-mm outer diameter) were inserted into the jugular vein, ascending aorta (through the left external carotid artery), and left ventricle (through the apical dimple) for drug administration and hemodynamic monitoring; the LV catheter was introduced after the heart had been exposed via a sternotomy and suspended in a pericardial cradle. Ventilation rate, volume, and inspired oxygen content were adjusted to maintain physiological values for arterial pO$_2$, pCO$_2$, and pH. Aortic and LV pressures were continuously monitored throughout the study.

$^{31}$P MRS measurements were performed using identical set up as in the validation experiments on skeletal muscle. Border zone measurements were obtained by suturing the coil directly to the LV epicardium over the peri-infarct region in animals from the MI and CELL groups, and remote-zone measurements were obtained by suturing the coil over a non-infarcted region; measurements in Normal animals were obtained with the coil positioned on the LV anterior wall. The proton signal from water, detected with the surface coil, was used to adjust the position of the animal in the magnet so that the coil was at the magnetic isocenter, thereby allowing the magnetic field to be homogenized and enabling acquisition of anatomic information for the planning of spatially localized $^{31}$P spectroscopy. All MR acquisitions were gated to both cardiac and respiratory cycle to minimize any motion-related artifacts. The complete myocardial MRS-MST protocol included the following 5 experiments/spectra: 1) Progressive saturation experiment with ATP$_{γ}$ saturated to measure the intrinsic T$_{1}$ of PCr; TR=6 sec, NEX=12. 2) Inversion recovery experiment with both PCr and Pi saturated to measure the intrinsic T$_{1}$ of ATP$_{γ}$; TR=4 sec., NEX=20. 3) Fully relaxed control spectrum without saturation to measure $M_{0,PCr}$ and $M_{0,ATPγ}$; TR=12 sec., NEX=20. 4) Spectrum with ATP$_{γ}$ saturated to measure $M_{ss,PCr}$; TR=6 sec., NEX=20. 5) Spectrum with both PCr and Pi saturated to measure $M_{ss,ATPγ}$; TR=6 sec., NEX=20. Spectra 3-5 were obtained with an interleaved acquisition pattern to minimize the influence caused by dynamic fluctuations of the MR signal. The total MRS data acquisition time for measurements taken under the baseline workload condition was ~30 min. After baseline assessments were complete, a high cardiac
workstate was induced via catecholamine stimulation (dobutamine/dopamine, 10 μg/kg per min, iv), and spectra 3-5 were acquired after hemodynamic parameters stabilized.

**Hemodynamic parameters and wall stress**

Hemodynamic parameters were measured during the in-vivo cardiac $^{31}$P MRS procedure via a polyvinyl chloride catheter (3 mm outer diameter) inserted into the left ventricle (through the apical dimple). Wall stress was calculated from the cardiac MRI and hemodynamic data according to the Laplace model:\textsuperscript{26}

$$\text{Systolic Wall stress} = \frac{\text{LV systolic pressure} \times \text{LV chamber radius}}{(2 \times \text{LV thickness})}$$

**Myocardial blood flow**

Myocardial blood flow was measured during the open-chest $^{31}$P MRS experiment. The detailed methods have been published previously.\textsuperscript{27, 28} Briefly, 1X10$^6$ fluorescently-labeled microspheres (Invitrogen) were re-suspended in 5 mL heparinized saline and injected into left atrium at a rate of 0.5 mL/sec. A reference sample of arterial blood (10 mL) was withdrawn from the ascending aortic catheter at a rate of 10 mL/min beginning 10 sec before microsphere injection. After sacrifice, LV samples (~1 gram) and the reference blood sample were lysed and analyzed for fluorescence intensity. The myocardial blood flow was calculated according to the following formula:

$$\text{Blood flow (mL/gram per min)} = \frac{\text{tissue fluorescence intensity/reference blood fluorescence intensity}}{\text{weight of tissue}} \times 10 \text{ mL/min}.$$  

**hiPSC-VC engraftment rate**

hiPSC-VC engraftment was evaluated by immuno-staining for GFP expression; 4 hearts were evaluated at each time point. After sacrifice, the LV was sectioned into 6 short-axis rings (~1-cm thickness) from the base to the apex as previously described,\textsuperscript{17} and each ring was sectioned into 12 blocks as indicated by the coronary anatomy; then, every second block from every second ring was paraffin embedded and sequentially cut into 7-um thick sections. GFP$^+$ cells were counted in slides from every 20 sections, and 10 high magnification view evaluations were averaged. A total of 5 slides were evaluated from each sample block to calculate the mean GFP$^+$ cell density (GFP$^+$ cells per cm$^2$)
for each block; then, the total GFP$^+$ cell count per block was estimated as: (mean GFP cell density per block)$^{3/2} \times$ volume of sample block, where the exponent (3/2) represents the conversion from surface density to volume density. The total number of engrafted cells was calculated as the sum of GFP$^+$ cell counts for all blocks multiplied by 4 (because only every second block from every second short-axis ring was evaluated). The GFP$^+$ cell engraftment rate was calculated as the total number of engrafted cells divided by the total number of cells administered (4 million).

Vascular structure
LV tissue samples (~1 cm$^3$) were embedded in Tissue-TEK OCT compound (Sakura Finetek, Zoeterwoude, The Netherlands) and then cryostat sectioned into 10-um thicknesses. Slides were immuno-stained for the presence of CD31 and SMA to visualize the vascular structure. Microscopic images (200X) were randomly taken across the stained area in a double-blind fashion. A total of 5 slides per sample, 2 samples per region (remote and border-zone) per animal, and 4 animals per experimental group were analyzed. Vascular density was expressed as the number of CD31$^+$ vessels per unit area (mm$^2$) and the number of SMA$^+$ vessels per unit area (mm$^2$).

Endogenous progenitor-cell activation and myocardial regeneration
BrdU (10 mg/kg per day) was injected into 3 randomly selected animals from each experimental group for the first 7 days after surgery. After sacrifice, LV tissue samples (~1 cm$^3$) were paraffin embedded and sequentially cut into 5-um transmural sections. Every 20$^{th}$ section was immuno-stained as previously described$^{29}$ for alpha sacromeric actin ($\alpha$SA) to visualize cardiomyocytes, for c-kit expression to identify progenitor cells, and for BrdU incorporation to identify proliferating cells. Progenitor-cell activation was evaluated by determining the c-kit$^+$ cell density (per unit area) and progenitor-cell proliferation was evaluated by determining the percentage of c-kit$^+$ cells that were also positive for BrdU incorporation. Cardiomyocytes and BrdU$^+$ cardiomyocytes were counted in every 20$^{th}$ slides, and sections with BrdU$^+$ myocytes were re-evaluated in consecutive sections to rule out the possibility of overlapping cells; cardiomyocyte
proliferation was evaluated by determining the percentage of cardiomyocytes that were positive for BrdU incorporation. Quantification of progenitor cells and myocytes were based on immunostaining of 5 slides per sample block, 2 blocks per heart and 3 hearts per group per time point of 1 week and 4 weeks. At least 10 high power images were randomly taken per section for histological analysis. For quantification of cardiomyocyte size, the images from border zone of CELL group at 4-week post surgery were analyzed. The mean cross-section diameter for BrdU\textsuperscript{+} cardiomyocytes were compared to that of BrdU\textsuperscript{-} cardiomyocytes from the same region.
SUPPLEMENTAL RESULTS

Pulses and pulse sequences for magnetization saturation transfer (MST) experiments

The MST experiments were performed by using a BISTRO pulse sequence to ensure frequency-selective elimination of magnetization in the presence of B1-inhomogeneity from the surface coil. (Supplemental Figure 1A).\textsuperscript{24, 25} Spin magnetization was excited with frequency-selective hyperbolic secant (HS) pulses of varying power levels (Figure 2B-C, red curves) and subsequently eliminated with de-phasing gradients. Double saturation was achieved by creating a composite pulse from two HS pulses with different excitation frequencies (Supplemental Figure 1B-C, blue curves). The magnetization response to the composite pulse is shown in Figure 3 (blue curve), where two excitation bands with distinct frequencies were observed (Supplemental Figure 1D a and b). A larger bandwidth was used for excitation frequency b to compensate for any change in the chemical shift of Pi that could result from variations of pH.

Indirect \textsuperscript{31}P MRS-MST method for measuring ATP turnover rates in vivo: validation in skeletal muscle

Our novel indirect \textsuperscript{31}P MRS-MST method for measuring in-vivo cardiac ATP turnover rates was validated with experiments performed in swine skeletal muscle (n=5, Supplemental Figure 2 and Table 1). The most essential step of the novel approach is the double saturation experiment to measure the total ATP utilization flux (ATP$\rightarrow$PCr and ATP$\rightarrow$Pi). Therefore, in order to validate the novel approach, we examined on whether the double saturation experiment could accurately measure the total ATP utilization flux through both PCr and Pi. The T\textsubscript{1} s for PCr, ATP\textsubscript{γ}, and Pi were measured with inversion-recovery experiments; saturation pulses were applied to ATP\textsubscript{γ} alone (Supplemental Figure 2A) or to both PCr and Pi (Supplemental Figure 2B) throughout the inversion-recovery experiment to decouple the chemical exchange and longitudinal relaxation processes. The apparent T\textsubscript{1} s (T\textsubscript{1} \textsuperscript{app}, Supplemental Figure 2C) were obtained from exponential fits of the MR data and used to calculate the intrinsic T\textsubscript{1} s for PCr (3.2±0.1 sec), ATP\textsubscript{γ} (1.0±0.1 sec), and Pi (6.1±0.2 sec) via equation [10]. Typical steady-state MST spectra of skeletal muscle are displayed in Supplemental Figure 2D.
The declines in PCr and Pi that occurred in response ATP\textsubscript{γ} saturation (i.e., the difference between spectra d2 and d1) were used to calculate the forward rate constants for the PCr→ATP ($k_{\text{PCr→ATP}}=0.22\pm0.01\ \text{s}^{-1}$) and Pi→ATP ($k_{\text{Pi→ATP}}=0.04\pm0.01\ \text{s}^{-1}$) reactions via equation [8], and the loss of ATP\textsubscript{γ} magnetization in response to simultaneous PCr and Pi saturation (i.e., the difference between spectra d2 and d3) was used to calculate the reverse rate constant for the combined ATP→PCr and ATP→Pi reactions ($k_{\text{ATP, tot}}=1.09\pm0.05\ \text{s}^{-1}$) via equation [17] (Supplemental Table 1). Under steady state condition, the ATP level is maintained, which means that the total ATP production rate must equal the utilization rate. The total ATP production rate is calculated by adding the flux of PCr→ATP and Pi→ATP reactions, and is measured with conventional MST approach with saturation on ATP\textsubscript{γ}. The total ATP utilization rate (ATP→PCr and ATP→Pi) is measured with double saturation experiment. As shown in Supplemental Figure 2 E, the two measurements match each other perfectly, suggesting the validity of the novel approach.

**Merits of the indirect \textsuperscript{31}P MRS-MST approach**

Conventional approaches for measuring ATP→Pi kinetics have not been used successfully for routine in-vivo assessments of cardiac ATP metabolism\textsuperscript{4, 5, 30}. Robitaille and colleagues used the conventional MRS-MST method to monitor the cardiac ATP turnover rate during global myocardial ischemia, when myocardial Pi increased to measurable levels, but measurements in healthy hearts or hearts with regional ischemia have been problematic\textsuperscript{31}. Portman determined that in lamb hearts, the unidirectional Pi→ATP rate constant is $0.49\pm0.09\ \text{s}^{-1}$ and that the Pi/ATP ratio is $0.4$\textsuperscript{32}, which suggests that $k_{\text{ATP→Pi}}$ is $\sim0.2\ \text{s}^{-1}$ assuming that ATP metabolism is in equilibrium during steady state. This estimated result is consistent with our measurements in uninjured swine hearts under baseline workload conditions ($k_{\text{ATP→Pi}}=0.17\pm0.02\ \text{s}^{-1}$), but the author acknowledged that the experiments were limited by an extended scan time and a poor signal-to-noise ratio for quantification of Pi.

The relationship between scan time and the signal-to-noise ratio of an MR spectrum is dependent on $T_1/M_0^2$, which suggests that the total scan time needed to quantify Pi
levels in swine skeletal muscle would be ~300-fold greater than the time needed to quantify PCr \( (M^2_{0,PCr}/M^2_{0,Pi} \times T_{1,PCr}/T_{1,PCr}) \) and ~50-fold greater than the time needed to quantify \( \text{ATP}^\gamma \) \( (M^2_{0,\text{ATP}^\gamma}/M^2_{0,Pi} \times T_{1,\text{ATP}^\gamma}/T_{1,\text{ATP}^\gamma}) \) with the same accuracy (Supplemental Table 1). Thus, our novel indirect \(^{31}\text{P}\) MRS-MST method dramatically reduces the scan time and improves the signal-to-noise ratio by eliminating the need to quantify Pi levels. The total scan time, including the measurement of \( T_1 \), is ~30 min, and if the \( T_1 \)s are already known (as when the same heart is re-evaluated under different workload conditions), the scan time needed for the three-spectra MST experiment is only 8 min. These advantages are likely to be relevant for investigations of many organs and physiological systems, because even in tissues where Pi can be quantified, such as the brain and skeletal muscle, it is much less abundant, and has a much longer \( T_1 \), than PCr or ATP. \(^4\) We have also recently developed a \( T_1^{\text{nom}} \) method that enables the ATP turnover rate to be accurately quantified under partially relaxed conditions, thereby reducing the scan time of the MST experiment by up to an order of magnitude. \(^21\) The \( T_1^{\text{nom}} \) technique is readily compatible with our new indirect 31P MRS-MST approach and, consequently, could reduce the scan time even further.
### Supplemental Table 1. $^{31}$P MST-MRS measurement data on swine skeletal muscle

<table>
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<th>n=5</th>
<th>PCr/ATP</th>
<th>Pi/ATP</th>
<th>$T_{1,PCr}^{\text{int}}$ (sec)</th>
<th>$T_{1,ATP}^{\text{int}}$ (sec)</th>
<th>$T_{1,Pi}^{\text{int}}$ (sec)</th>
<th>$k_{\text{PCr} \rightarrow \text{ATP}}$ (sec$^{-1}$)</th>
<th>$k_{\text{Pi} \rightarrow \text{ATP}}$ (sec$^{-1}$)</th>
<th>$k_{\text{ATP} \rightarrow \text{Pi}}$ (sec$^{-1}$)</th>
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<tbody>
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<td>value</td>
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<td>0.41±0.04</td>
<td>3.2±0.1</td>
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<td>6.1±0.2</td>
<td>0.22±0.01</td>
<td>0.04±0.01</td>
<td>0.02±0.02</td>
</tr>
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Supplemental Figure 1. Magnetization saturation transfer (MST) experiments.  

Panel A, pulse sequence of BISTRO-based magnetization saturation. $t_{\text{sat}}$ and $t_{\text{acq}}$ represent the time of saturation and acquisition, respectively. Panels B-C, RF pulses used for single (red) and double (blue) saturation in the MST experiments. Panels B and C showed the relative amplitude and the phase of the hyperbolic secant-based RF pulses. Panel D showed the response of spin magnetization to RF pulses excitation with various power levels. The single saturation RF pulse (red) resulted in a single excitation frequency (a) whereas the double saturation RF pulse (blue) resulted in two excitation frequencies (a and b). Note that the excitation band b (for Pi saturation) has a wider bandwidth than a in order to compensate the possible change of Pi chemical shift due to change of pH values.
Supplemental Figure 2. $^{31}$P MRS-MST experiments on swine skeletal muscle.

Panels A-C, measurements of apparent $T_1$s for PCr and Pi (panel A, with saturation on ATPγ) and ATPγ (panel B, with saturation on both PCr and Pi) using inversion-recovery method. The magnetizations were plotted against inversion time and fit with an exponential model to retrieve the corresponding apparent $T_1$s (panel C). Panel D, steady-state magnetization saturation transfer experiments to measure the rate constants of $k_{PCr\rightarrow ATP}$, $k_{Pi\rightarrow ATP}$ and $k_{ATP,tot}$ based on equations [8] and [17], respectively. d1: ATPγ-saturated spectrum to measure $M_{ss,PCr}$ and $M_{ss,Pi}$. d2: control spectrum without saturation to measure the $M_{0,PCr}$, $M_{0,ATPγ}$ and $M_{0,Pi}$. d3: both Pi- and PCr-saturated spectrum to measure the $M_{ss,ATPγ}$. Panel E, plot of normalized flux (flux/[ATP]) for total ATP production and utilization. The total ATP production consists of two reactions: PCr $\rightarrow$ ATP and Pi $\rightarrow$ ATP, and are measured by conventional MST approach with saturation on ATPγ. The total ATP utilization flux is measured by double saturation on both PCr and Pi. The results from 5 independent measurements showed no statistical difference between the two measurements.
Supplemental Figure 3. **Panel A**, numerous hiPSC-VCs were detected at 1 WK post surgery by GFP staining (green). Co-staining with BrdU (red) indicated active proliferation of these transplanted cells in vivo. **Panel B**, transplanted hiPSC-VCs contributed to neo-vascularization by generating new vessels. Arrows indicate vessels stained positive for GFP (green) and human specific CD31 (hCD31, red) inside the fibrin patch.
Supplemental Figure 4. Activation of c-kit⁺ progenitor cells in response to myocardial injury and hiPSC-VCs transplantation. Panels A-F, Representative immuno-staining images from Normal (A and D), infarct area of MI (B and E) and CELL (C and F) hearts at both 1 week (A-C) and 4 weeks (D-F) post surgery. Cardiac progenitor cells were identified by c-kit staining (green), cardiomyocytes were identified by alpha sacromeric actin staining (αSA, yellow), and cycling cells were identified by BrdU incorporation (red). Arrowheads indicate c-kit⁺/BrdU⁺ cells whereas arrows indicate c-kit⁺/BrdU⁻ cells.
SUPPLEMENTAL VIDEO LEGENDS

**Supplemental Video 1.** Illustration of an epicardial fibrin patch to deliver the mixture of 4 million hiPSC-VCs on the surface of the LV infarct.

**Supplemental Video 2.** Representative cardiac cine imaging of an aged-matched NORMAL heart.

**Supplemental Video 3.** Representative cardiac cine imaging of an MI heart at 4 weeks post infarction.

**Supplemental Video 4.** Representative cardiac cine imaging of a CELL heart at 4 weeks post infarction.
SUPPLEMENTAL REFERENCE


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