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relations between myosin mass transfer were evident.

Figure 5. Observed 1,0 reflection intensity change, calculated myosin interfilament spacing ($d_{1,0}$), myosin mass transfer index (equatorial intensity ratio $I_{1,0}/I_{1,1}$) and simultaneously acquired LV pressure acquired over several consecutive cardiac cycles in an iPSC-CM sheet of a transplant heart. Significant 1,0 myosin reflections were only evident for part of the cardiac cycle due to heart movement. When significant actin-myosin reflections were evident the shift in myosin mass towards actin (decrease in intensity ratio) coincided with the rapid increase in LV pressure during systole showing synchronized contraction of the iPSC-CMs in the sheet. Arrows indicate timing of end diastole.

Figure 6. Dsred-labelled transplanted iPSC-CMs expressed clear myosin-positive sarcomeres as shown in the representative confocal micrograph **(A)**. The sarcomere of the transplanted iPSC-CMs consisted of myosin and sarcomeric actin **(B)**. Distribution of the Cx43 did not show the typical intercalated disks in the transplanted iPSC-CMs **(C)**. The scale bar indicates ten micrometers. The iPSC-CMs *in vitro* showed the cardiac myocyte-like sarcomeres with less dense mitochondrial structures (arrow) as shown in the representative electron micrograph **(D)**. *In vivo* transplanted iPSC-CMs showed clear desmosome structure between the cells (arrow heads, **E**). Mitochondria of the transplanted iPSC-CMs *in vivo* gradually showed a dense structure at day 3 **(E)** and then at day 7 **(F)**. n=5 each.

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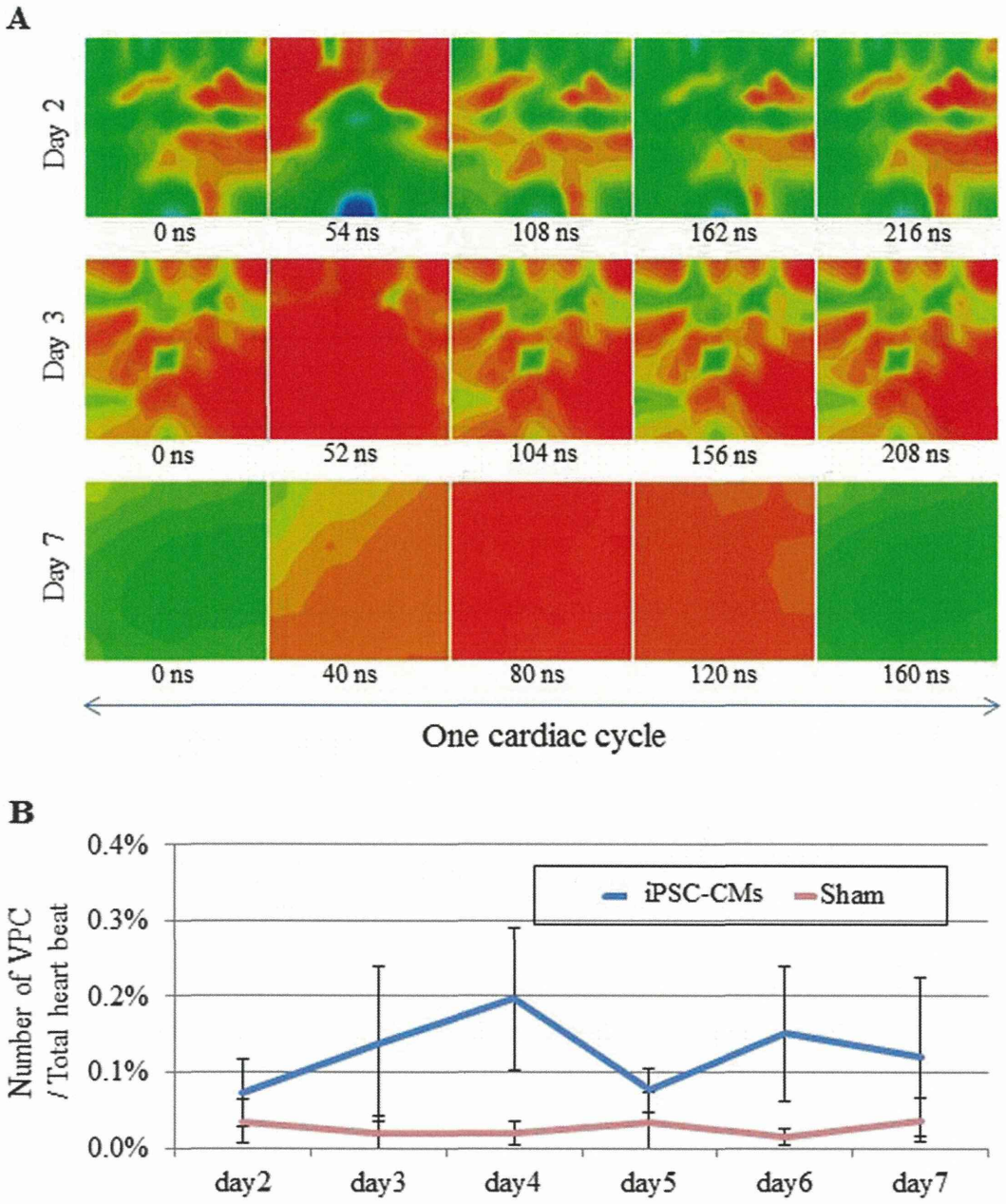

Figure 1

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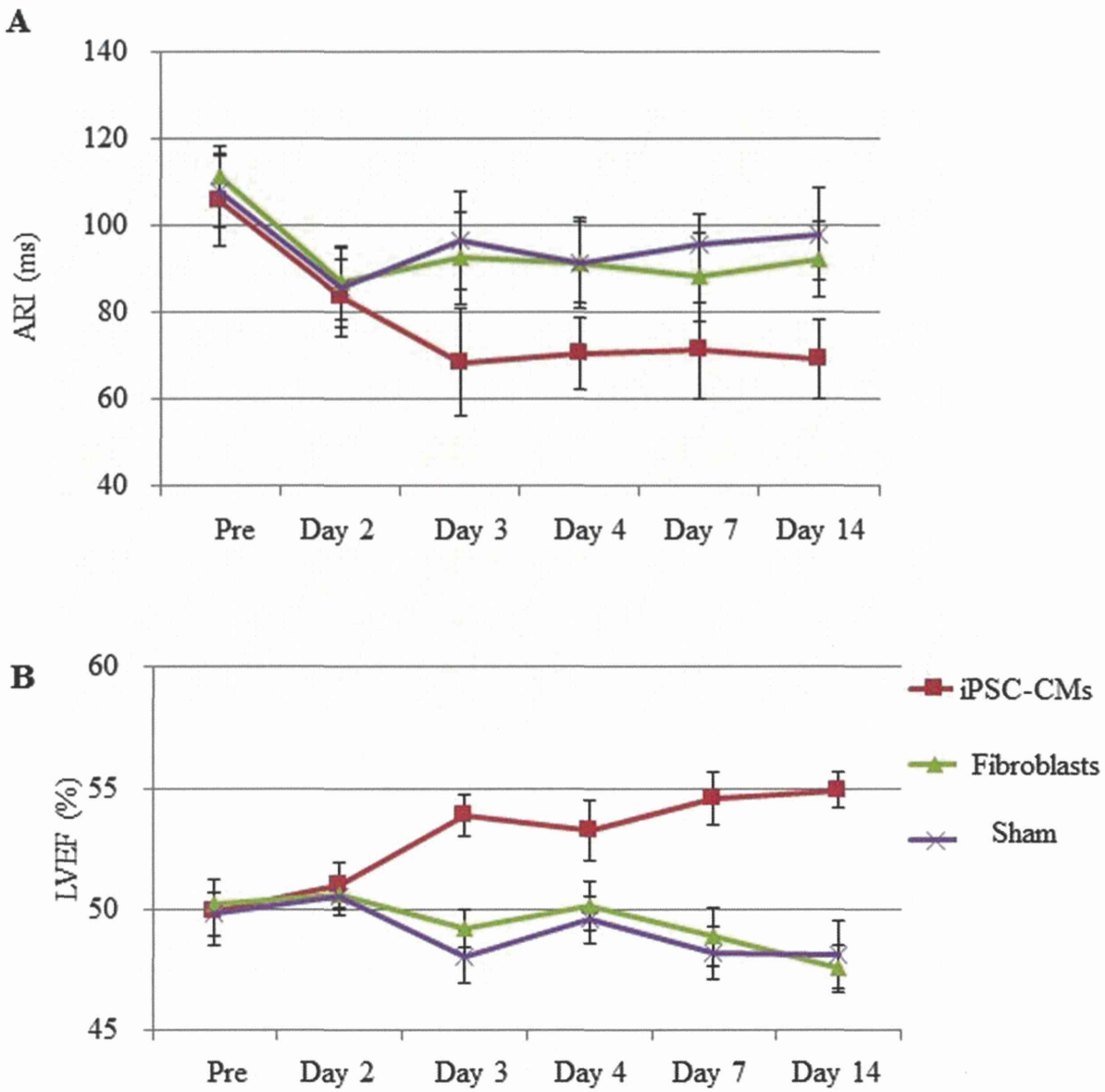

Figure 2

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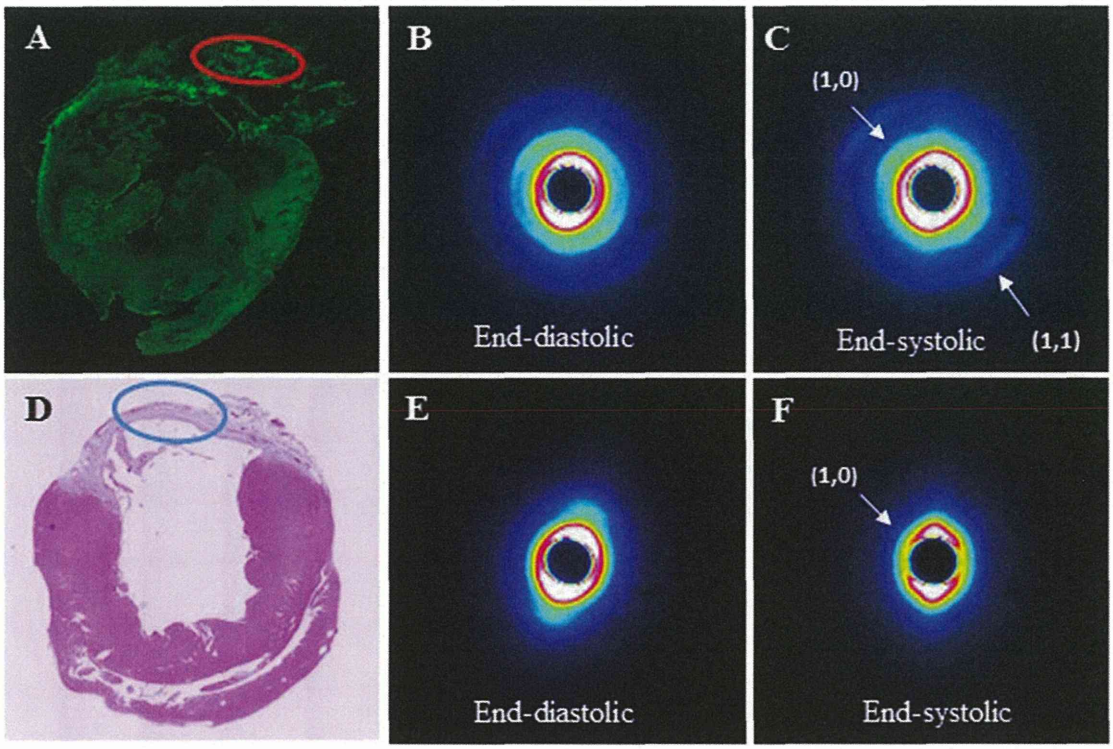

Figure 3

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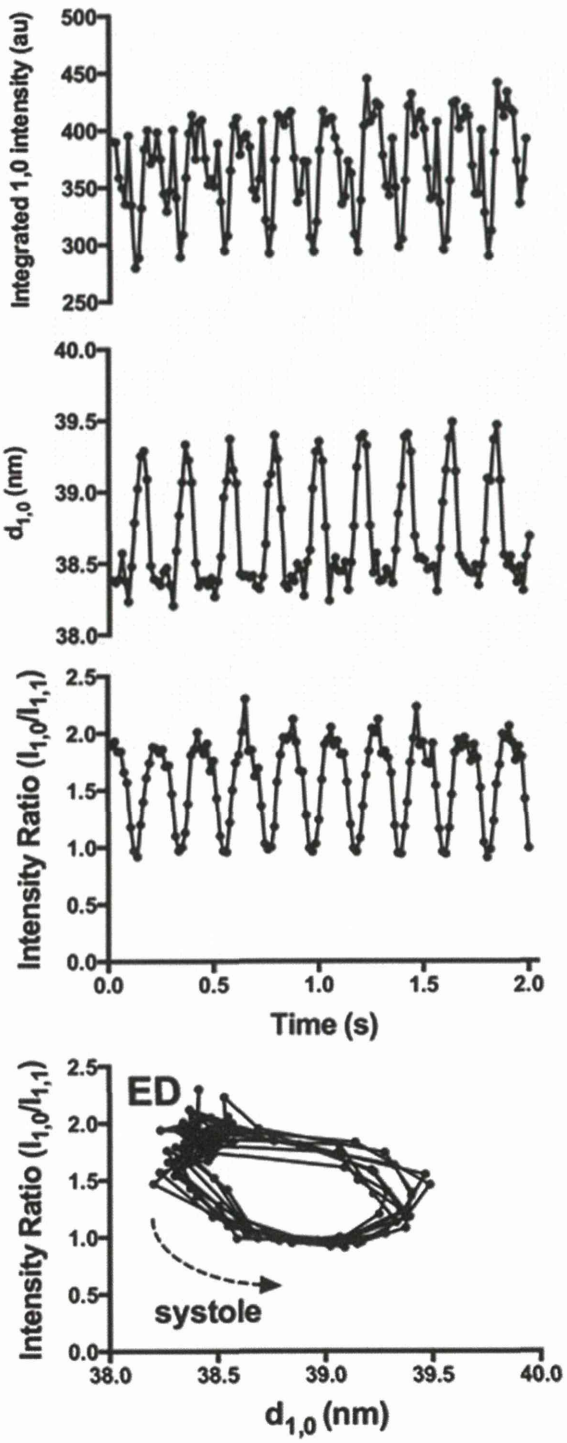

Figure 4

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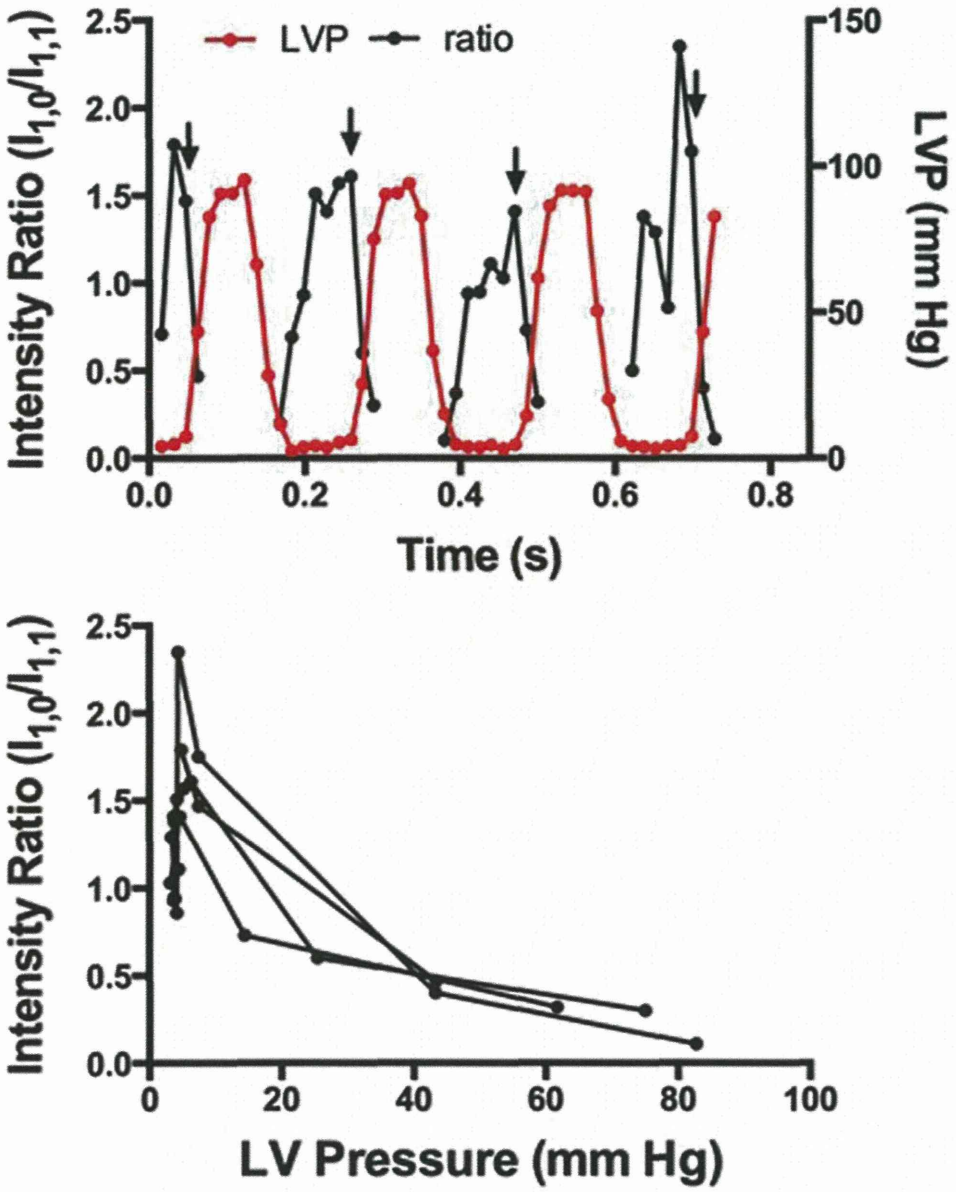

Figure 5

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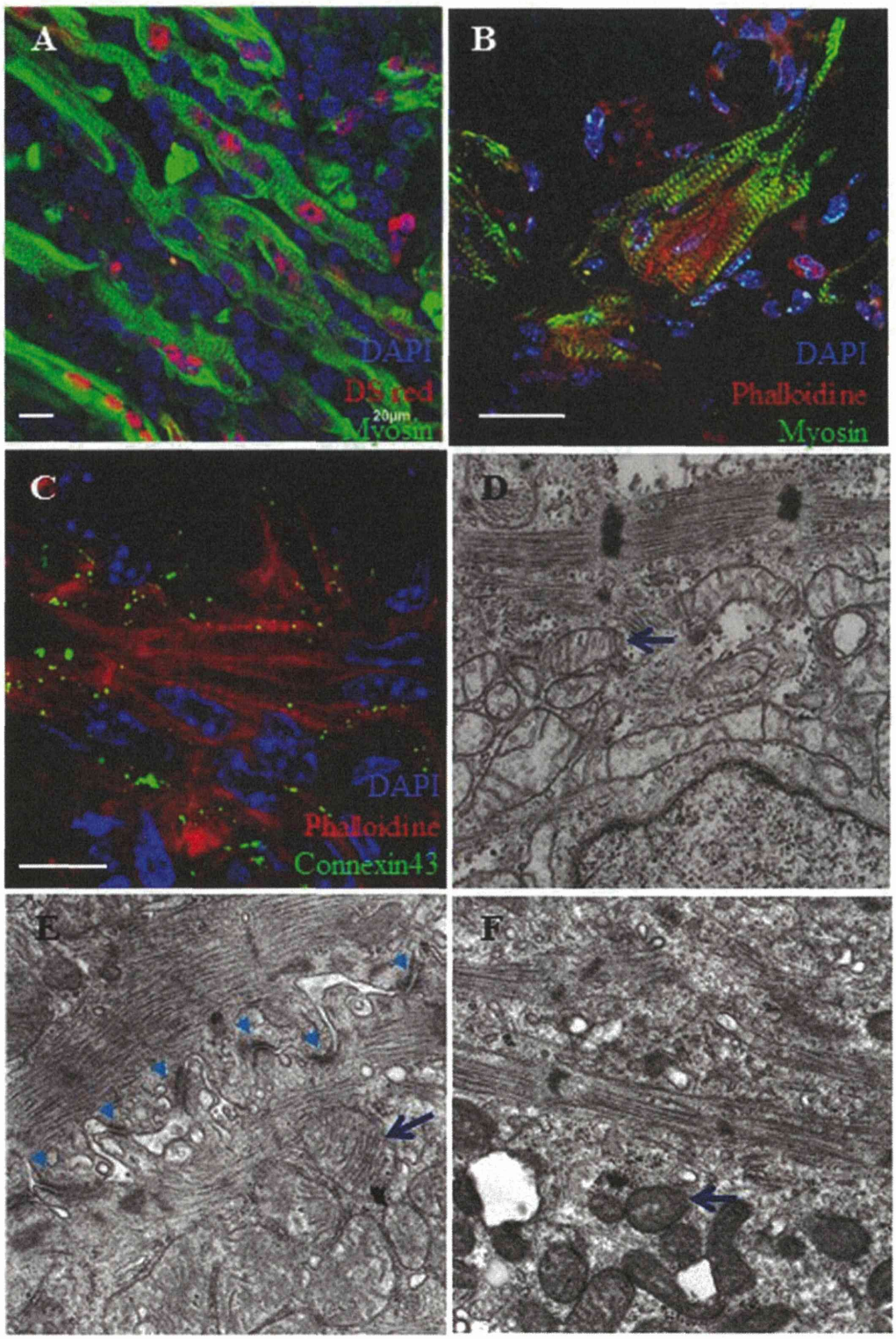

Figure 6

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Cell-sheet Therapy With Omentopexy Promotes Arteriogenesis and Improves Coronary Circulation Physiology in Failing Heart

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Cell-sheet transplantation induces angiogenesis for chronic myocardial infarction (MI), though insufficient capillary maturation and paucity of arteriogenesis may limit its therapeutic effects. Omentum has been used clinically to promote revascularization and healing of ischemic tissues. We hypothesized that cell-sheet transplantation covered with an omentum-flap would effectively establish mature blood vessels and improve coronary microcirculation physiology, enhancing the therapeutic effects of cell-sheet therapy. Rats were divided into four groups after coronary ligation; skeletal myoblast cell-sheet plus omentum-flap (combined), cell-sheet only, omentum-flap only, and sham operation. At 4 weeks after the treatment, the combined group showed attenuated cardiac hypertrophy and fibrosis, and a greater amount of functionally ($CD31^+$/$lectin^+$) and structurally ($CD31^+$/α-SMA^+) mature blood vessels, along with myocardial upregulation of relevant genes. Synchrotron-based microangiography revealed that the combined procedure increased vascularization in resistance arterial vessels with better dilatory responses to endothelium-dependent agents. Serial ^{13}N-ammonia PET showed better global coronary flow reserve in the combined group, mainly attributed to improvement in the basal left ventricle. Consequently, the combined group had sustained improvements in cardiac function parameters and better functional capacity. Cell-sheet transplantation with an omentum-flap better promoted arteriogenesis and improved coronary microcirculation physiology in ischemic myocardium, leading to potent functional recovery in the failing heart.

Received 27 August 2014; accepted 16 November 2014; advance online publication 13 January 2015. doi:10.1038/mt.2014.225

INTRODUCTION

Heart failure following myocardial infarction (MI) is a major cause of death and disability worldwide. Despite advances in drug and device therapy, recovery of cardiac function and prevention of transition to heart failure in MI patients remain unsatisfactory, indicating the need for development of novel therapeutic alternatives.[1] Myocardial regenerative therapy with cell-sheet transplantation has been shown to induce angiogenesis via paracrine effects in a chronic MI model.[2,3] However, the proangiogenic effect of the stand-alone cell-sheet treatment may be insufficient to fully relief ischemia in the chronic MI heart that involves a large territory of the left ventricle (LV), since the coronary inflow of the ischemic/infarct myocardium is dependent upon collateral arteries from other territories.[4,5] In addition, microvascular dysfunction is present in critical chronic MI heart across a wide range of the peripheral coronary tree.[6] This highlights the need for a comprehensive understanding of the mechanism of angiogenesis induced by a cell-sheet therapy in ischemic hearts.

For successful therapeutic neovascularization of ischemic tissues, it is essential to induce robust angiogenic responses (angiogenesis), and establish functionally and structurally mature arterial vascular networks (arteriogenesis) that show long-term stability and control perfusion.[5] Establishment of mature vessels is a complex process that requires several angiogenic factors to stimulate vessel sprouting and remodeling (endothelial tubulogenesis accompanied with a pericyte recruitment) of the primitive vascular network. Endothelial vasodilator function of coronary microvessels (resistance arterial vessels) is also an important determinant of myocardial perfusion in response to increased myocardial oxygen demand, playing a critical role in neovascular therapies.[6–8] The attenuated therapeutic effects observed in the previous clinical trials were caused by multiple factors including

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generation of unstable blood vessels that regress over time or functionally immature vessels accompanied with endothelial dysfunction in ischemic areas.[5,9]

The omentum (OM), historically used in surgical revascularization for patients with ischemic heart disease, is also known to release a number of angiogenic cytokines and attenuate inflammation.[10–14] In addition, the gastroepiploic artery involved in the OM-flap can play an important role as an extracardiac blood source with high perfusion capacity for developing effective collateral vessels for advanced coronary artery disease. We established a combination strategy of cell-sheet transplantation covered with a pedicle OM-flap in porcine models, allowing us to implant large numbers of cells and improve cell survival.[13,14] However, data are scarce regarding the therapeutic effects of such combined treatment on vessel maturity and coronary microcirculation physiology in ischemic territory. We hypothesized that cell-sheet transplantation with a pedicle OM-flap will better promote arteriogenesis and stabilize blood vessels in ischemic myocardium along with improved coronary microcirculation physiology, consequently enhancing the therapeutic effects of cell-sheet therapy. Herein, we focused on vessel maturation induced by cell-sheet therapy with an OM-flap and evaluated the physiological benefits in coronary microcirculation utilizing modern modalities such as *in vivo* synchrotron-based microangiography and positron emission tomography (PET).

RESULTS

Histological analysis of host myocardium

Four weeks after treatment, myocardial structural components, collagen accumulation and cardiomyocyte hypertrophy, were assessed by hematoxylin-eosin, Masson trichrome, and Periodic acid-Schiff staining (n = 11 for each group). LV myocardial structure was better maintained in the combined group as compared with the others (**Figure 1c**). The combined group had a significantly thickened anterior LV wall (anterior wall thickness, control 392 ± 31 versus combined 912 ± 34 versus sheet-only 688 ± 27 versus OM-only 500 ± 28 μm) (**Figure 1d**). That group also had a significantly attenuated collagen accumulation (percent fibrosis, 18 ± 1 versus 8 ± 4 versus 13 ± 6 versus 14 ± 1%, respectively) (**Figure 1e**) and cardiac hypertrophy (myocyte size, 23 ± 1 versus 16 ± 1 versus 20 ± 3 versus 21 ± 2 μm, respectively) (**Figure 1f**) in the peri-infarct regions (ANOVA $P < 0.001$ for all).

Gene expressions in peri-infarct myocardium during acute treatment phase

The myocardial gene expressions related to angiogenesis, vessel maturation, and anti-inflammation were analyzed at 3 days after each treatment using real-time PCR (n = 6 for each group). As compared to the others, the combined group showed substantially higher gene expressions of *vascular endothelial growth factor (VEGF)-A*, *VEGF receptor-1*, *VEGF receptor-2*, *Akt-1*, *platelet-derived growth factor (PDGF)-β*, *angiopoietin (Ang)-1*, *Tie-2*, *vascular endothelial (VE)-cadherin*, *platelet endothelial cell adhesion molecule (PECAM)-1*, and *stromal cell-derived factor (SDF)-1* in peri-infarct myocardium at the early stage of transplantation (**Figure 2**).

Vessel recruitment in transplanted cell-sheets and donor cell survial

To evaluate the effect of adding OM-flap to the cell-sheet therapy on the vessel recruitment (angiogenesis) in the transplanted area that should be related to the donor cell survival, we serially assessed the number of functional blood vessels with patent endothelial layers (CD31/lectin double-positive cells) in the transplanted area of the sheet-only and combined groups at 3, 7, and 28 days after each treatment (n = 6 for each group and each time point) (**Figure 3a–f**). At 3 days after treatment, in the sheet-only group, several blood vessels were just located at the border between the sheet and infarct area (**Figure 3a**), whereas a large number of functional vessels was detected proximal to the border between the cell-sheet and OM and within the sheet in the combined group (**Figure 3d**), suggesting that the cell-sheet received blood supply directly from the infarct myocardium and OM. Consequently, the combined group had greater numbers of functional blood vessels in the cell-sheet than the sheet-only group at any follow-up point, although both groups showed steady decrease in the number of vessels during the 28 days (**Figure 3g**).

The quantitative assessments of the donor (GFP-positive) cell presence were also serially performed to elucidate the donor cell dynamics in the sheet-only (**Figure 3a–c**) and combined (**Figure 3d–f**) groups. We traced the transplanted donor cells and found that there was no significant difference in the engrafted area at 3 days after transplantation between the groups, while the subsequent changes in each group were apparently distinctive (**Figure 3h**). During the 7days after the treatment, the amount of decrease in the engrafted area was substantially smaller in the combined group than that in the sheet-only group, resulting in 4.3-fold increased retention of donor cells in the former group. This led to the greater donor cell presence in the combined group persistently (at least until day 28), which was consistent with the amount of vessel recruitment in the cell-sheet.

Vessel remodeling and maturation in peri-infarct myocardium

We serially assessed neovascular vessel maturity in peri-infarct areas at 3 (n = 6 for each group) and 28 days (n = 11 for each group) after treatment (**Figure 4**). Vessel density and structural maturity were quantified as the number of CD31 positive and CD31/α-smooth muscle actin (SMA) double-positive vessels per mm^2, respectively. A maturation index was calculated as the percentage of CD31/α-SMA double-positive vessels to total vessel number. Functionally mature vessels with patent endothelial layers were assessed by lectin injection, which binds uniformly and rapidly to the luminal surface of endothelium, thus labeling patent blood vessels. Vessels positive for CD31 but negative for lectin were regarded as functionally immature and undergoing regression, or that had lost patency.[15,16]

In general, α-SMA signals were located at the outer edges of CD31 staining, indicating pericyte attachment to newly formed endothelium. Three days after treatment, there was no difference in number of CD31-positive cells among the groups, though the combined group showed a trend of greater number of functional blood vessels with patent endothelial layers (CD31/lectin double-positive) and structurally (CD31/α-SMA double-positive) mature vessels, with a higher maturation index (**Figure 4a–g**). Notably, the percentage without lectin staining (CD31$^+$/lectin$^-$) was significantly smaller in the combined group.

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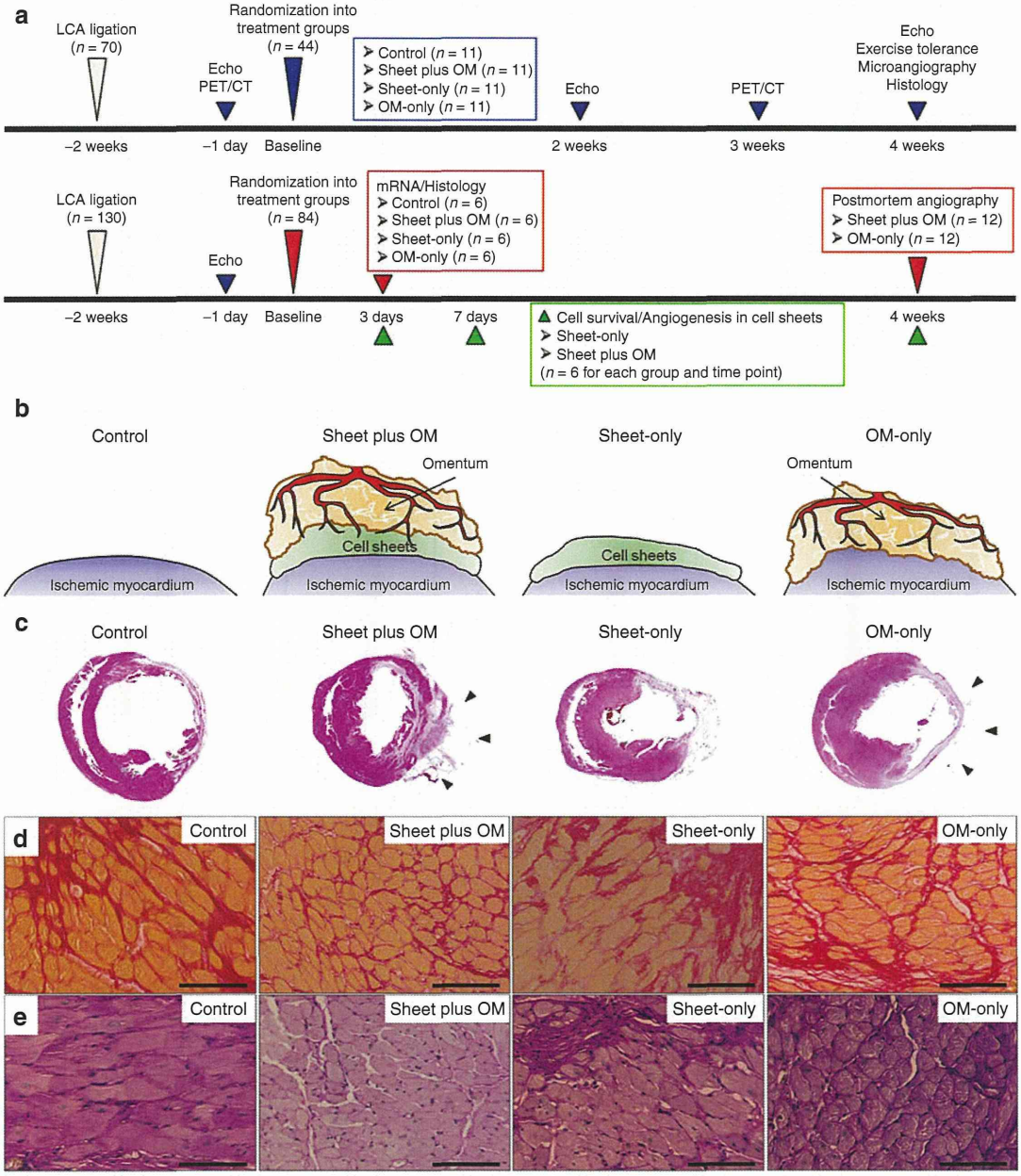

Figure 1 (**a**) Experimental protocols. (**b**) Procedural schemes for treatment groups. (**c**) Macroscopic images of HE-stained whole sections of the left ventricle and (**d**) anterior wall thickness (40×, scale bar = 1,000 μm). Black arrows indicate the omentum tissue. Photomicrographs of Sirius red- (**e**) and periodic acid-Schiff-stained (**f**) sections of peri-infarct myocardium (400×, scale bar = 100 μm) (n = 11 for each group).

The number of endothelial (CD31 positive) cells in the control and single treatment groups decreased with time, while that in the combined remained unchanged. Consequently, the angiogenic effects induced in the latter were more profound at 28 days after treatment, with a significantly greater amount of mature vessels (**Figure 4h–n**).

Number of resistance vessels and relative dilatory responses to endothelium-dependent stimulation in ischemic myocardium

To evaluate the effects of each treatment on microcirculation physiology in terms of relative dilatory responses to acetylcholine and dobutamine hydrochloride in the resistance vessels, synchrotron radiation microangiography was performed after 3 weeks after the treatment (control: n = 11, combined: n = 11, cell-sheet: n = 5, OM: n = 6). Using iodinated agents, coronary microcirculation in ischemic areas was clearly visualized in anesthetized closed-chest rats (**Figure 5a**). Vessel internal diameter (ID) at baseline (before agent administration) tended to decrease according to branching order and differed among the groups with larger first branching order arteries observed in the combined group (**Figure 5b**). Moreover, the combined group had a greater number of third and fourth branching order arterial vessels (resistance arterial vessels) at baseline (**Figure 5c**).