Skeletal myoblast sheet transplantation improves the diastolic function of a pressure-overloaded right heart

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Objective: The development of right ventricular dysfunction has become a common problem after surgical repair of complex congenital heart disease. A recent study reported that tissue-engineered skeletal myoblast sheet transplantation improves left ventricular function in patients with dilated and ischemic cardiomyopathy. Therefore myoblast sheet transplantation might also improve ventricular performance in a rat model of a pressure-overloaded right ventricle.

Methods: Seven-week-old male Lewis rats underwent pulmonary artery banding. Four weeks after pulmonary artery banding, myoblast sheet transplantation to the right ventricle was performed in the myoblast sheet transplantation group (n = 20), whereas a sham operation was performed in the sham group (n = 20).

Results: Four weeks after performing the procedure, a hemodynamic assessment with a pressure–volume loop showed a compensatory increase in systolic function in both groups. However, only the myoblast sheet transplantation group showed a significant improvement in the diastolic function: end-diastolic pressure (sham vs myoblast sheet transplantation, 10.3 ± 3.1 vs 5.0 ± 3.7 mm Hg; $P < .001$), time constant of isovolumic relaxation (11.1 ± 2.5 vs 7.6 ± 1.2 ms, $P < .001$), and end-diastolic pressure–volume relationship (16.1 ± 4.5 vs 7.6 ± 2.4 mL, $P < .005$). The right ventricular weight and cell size similarly increased in both groups. A histologic assessment demonstrated significantly suppressed ventricular fibrosis and increased capillary density in the myoblast sheet transplantation group in comparison with those in the sham group. Reverse transcription–polymerase chain reaction demonstrated an increased myocardial gene expression of hepatocyte growth factor and vascular endothelial growth factor in the myoblast sheet transplantation group but not in the sham group.

Conclusions: Skeletal myoblast sheet transplantation improved the diastolic dysfunction and suppressed ventricular fibrosis with increased capillary density in a rat model of a pressure-overloaded right ventricle. This method might become a novel strategy for the myocardial regeneration of right ventricular failure in patients with congenital heart disease.

Because of recent developments in diagnostic methods, the establishment of new surgical techniques, and improvements in perioperative management, patients with complex congenital heart disease (CHD) are today often able to survive to adulthood. However, even after a successful repair, right ventricular (RV) overload remains in some patients, in whom it impairs RV function and influences long-term mortality and morbidity.^{1–3} Chronic pressure overload is one of the major risk factors of RV dysfunction. In this

situation the right ventricle is hypertrophied and systolic function is initially preserved, whereas diastolic function gradually deteriorates.^{4,5} Prolonged exposure to excessive pressure overload results in irreversible RV failure. Clinically, the relationship between progressive fibrosis and RV function must be addressed.^{6–8}

Recently, cardiac regeneration therapy has provided a new treatment for end-stage heart failure, and skeletal myoblasts are currently thought to be a potential cell source.^{9–11} We developed a novel cell delivery system using temperature-responsive culture dishes,¹² and tissue-engineered cell sheets have been created without any scaffold, which maintains cell–cell interaction and extracellular matrix while avoiding any inflammatory reaction, and with improved cell survival.¹³ Skeletal myoblast sheet transplantation (MST) has been shown to improve left ventricular (LV) contractility in several animal models of LV failure.^{14–16} Otherwise, it is unclear whether MST can also affect the right ventricle, especially pressure-induced RV dysfunction. Hence this study assessed whether MST could improve RV function in rats after damage caused by pressure overload.

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Abbreviations and Acronyms

BW	= body weight
CFR	= coronary flow reserve
EDPVR	= end-diastolic pressure–volume relationship
Ees	= end-systolic elastance
ESPVR	= end-systolic pressure–volume relationship
GAPDH	= glyceraldehyde-3-phosphate dehydrogenase
HGF	= hepatocyte growth factor
IVS	= intraventricular septum
LV	= left ventricular
MS	= myoblast cell sheet
MST	= myoblast sheet transplantation
PA	= pulmonary artery
PAB	= pulmonary artery banding
PRSW	= preload recruitable stroke work
RT-PCR	= reverse transcription–polymerase chain reaction
RV	= right ventricular
SW	= stroke work
VEGF	= vascular endothelial growth factor

MATERIALS AND METHODS**Animal Care**

All experimental procedures and protocols used in this investigation were reviewed and approved by the institutional animal care and use committee and are in accordance with the National Institutes of Health “Guide for the care and use of laboratory animals” (National Institutes of Health publication no. 85-23, revised 1996).

Creation of Chronic RV Pressure Overload

A rat model of pulmonary artery banding (PAB) was established to create chronic RV pressure overload. Seven-week-old male Lewis rats (180–210 g) were anesthetized with an intraperitoneal injection of ketamine hydrochloride (50 mg/kg) and xylazine (5 mg/kg) and ventilated by using a volume-controlled respirator (2 mL, 60 cycles/min) with room air. A left thoracotomy was performed at the fourth intracostal space, and the main pulmonary artery (PA) was carefully exposed. As previously reported,¹⁷ a 19-gauge injection needle (outer diameter, 1.1 mm) was placed alongside the PA, and a 3-0 polyester suture was tied tightly around the PA and the needle. Next, the needle was rapidly removed, and then a fixed diameter was set for the PA. Thereafter, the thorax was closed in layers, and the ventilator setting was changed (90 cycles/min) for half an hour to reduce the respiratory load.

Skeletal Myoblast Sheet Preparation

Creation of myoblast cell sheets (MSs) with temperature-responsive culture dishes (UpCell; Cellseed, Tokyo, Japan) was done according to previous reports.^{14–16} Briefly, skeletal muscle was harvested from the hind legs of 4-week-old syngeneic rats. The purified myoblasts were incubated on 35-mm UpCell dishes at 37°C, with the cell numbers adjusted to 3×10^6 per dish. After 12 to 18 hours, the dishes were moved to a refrigerator set at 20°C and left there for 30 minutes. During that time, the MSs detached spontaneously from the surfaces. Each sheet measured from 10 to 15 mm in diameter.

Skeletal MST

Four weeks after PAB, a second left thoracotomy was performed at the fifth intracostal space after achievement of general anesthesia. After opening the pericardium, the RV anterior wall was exposed. Two MSs were grafted onto each anterior wall of the right ventricle in the MST group (n = 20), or a sham operation was performed in the sham group (n = 20). The pericardium was closed linearly before the thorax was closed to prevent the dislocation of MSs. In addition, age-matched rats that did not undergo surgical intervention were also prepared as a control group (n = 20).

Hemodynamic Study and Data Analysis

Four weeks after the MST or sham operation, 10 rats in each group were anesthetized and ventilated again and were set on the blanket warmer to maintain body temperature. A median sternotomy was performed, and the pericardium was opened carefully to minimize hemorrhaging. A silk thread was placed under the inferior vena cava just above the diaphragm to change the RV preload. After purse-string sutures were attached with 7-0 polypropylene, the conductance catheter (Unique Medical Co, Tokyo, Japan) was inserted through the RV apex toward the pulmonary valve along the longitudinal axis of the RV cavity and then fixed. A Miller 1.4F pressure-tip catheter (SPR-719; Millar Instruments, Houston, Tex) was also inserted from the RV anterior wall and fixed. For better volume measurement, a 1-mm curve was added to the original standard straight conductance catheter to fit the complex RV geometry. The position of the conductance catheter was determined by observing the pressure and segmental volume signals with the appropriate phase relationships. The conductance system and the pressure transducer controller (Integral 3 [VPR-1002], Unique Medical Co) were set as previously reported.¹⁸ Pressure–volume loops and intracardiac electrocardiograms were monitored online, and the conductance, pressure, and intracardiac electrocardiographic signals were analyzed with Integral 3 software (Unique Medical Co).¹⁸

Under stable hemodynamic conditions, the baseline indices were initially measured, and then the pressure–volume loop was drawn during inferior vena caval occlusion and analyzed (Figure 1). Finally, the conductivity of the sampled blood was measured with a small (0.1 mm) cuvette, and the parallel conductance volume was measured with the hypertonic saline dilution method to obtain the absolute volumes.¹⁹

The following indices were calculated as the baseline RV function: heart rate, end-systolic pressure, end-diastolic pressure, dP/dt_{max} , dP/dt_{min} , and the time constant of isovolumic relaxation (τ). The following relationships were determined by means of pressure–volume loop analysis as load-independent measures of RV function: end-systolic pressure–volume relationship (ESPVR), end-diastolic pressure–volume relationship (EDPVR), and preload recruitable stroke work (PRSW).

The ESPVR is linear, and it can be characterized by a slope (end-systolic elastance [Ees]) and a volume axis intercept (V_0), so that $P_{es} = E_{es}(V_{es} - V_0)$, where P_{es} and V_{es} are the end-systolic pressure and volume, respectively.²⁰

In contrast, the EDPVR is intrinsically thought to be nonlinear. The relationship between the end-diastolic pressure (P_{ed}) and volume (V_{ed}) can be fitted to the monoexponential, so that $P_{ed} = P_0 + b e^{K_v V_{ed}}$, where P_0 is the pressure asymptote (generally close to 0 mm Hg), b is a constant, and K_v is the variable represented as a ventricular stiffness property.²¹

The relationship between ventricular stroke work (SW) and end-diastolic volume (V_{ed}) is represented as PRSW. PRSW is thought to be a suitable parameter of the contractile state and fitted to the following equation: $SW = K(V_{ed} - V_0)$, where K is a constant as a potential measure of intrinsic myocardial performance independent of loading, geometry, and heart rate.²²

Histopathologic Analysis

The other 10 rats in each group were killed 4 weeks after the sham or MST operation for histologic analysis, reverse transcription–polymerase chain reaction (RT-PCR), and blood sampling. The hearts were quickly removed, and the ventricles were dissected free of atrial tissue and large

FIGURE 1. Representative pressure–volume loops of the control (A), sham (B), and myoblast sheet transplantation (MST; C) groups under different loading conditions. The slope of the end-systolic pressure–volume relationship is displayed as a black straight line. The correlation of the end-diastolic pressure–volume relationship is displayed as a green monoexponential curve. RVP, Right ventricular pressure; RV, right ventricle.

blood vessels. The right ventricle was carefully separated from the left ventricle and intraventricular septum (IVS). The fresh ventricular tissues were immediately blotted dry and weighted separately to determine the degree of RV hypertrophy based on 2 parameters: RV wall weight/body weight (RV/BW) and RV wall weight/LV and IVS wall weight (RV/LV+IVS).

Tissue specimens were obtained from the endocardium, the midwall, and the epicardium of the RV anterior wall in cross-sections, cut into 5- μ m-thick sections, and stained with hematoxylin and eosin for morphologic analysis, including measurement of RV wall thickness, periodic acid–Schiff staining to measure the short-axis length of the RV myocardial cell, Factor VIII–related antigen staining (Dako EPOS anti-human Von Willebrand factor/HRP; Dako Cytomation, Glostrup, Denmark) to quantify capillary vascular density, and Masson trichrome staining for determination of the amount of interstitial and myocardial fibrosis. The percentage of interstitial and myocardial fibrosis were assessed by a computer-based method^{23,24} with the use of a software filter (Mac Scope Software; MITANI Corp, Tokyo, Japan), which can recognize the distinct color shades. The number of pixels of the blue-stained collagen area was calculated, then divided by the total number of pixels in a field. Each 3 fields of the endocardial, epicardial, and mid layers of the RV wall per slide were analyzed and then averaged.

RT-PCR

Total RNA was isolated from the stored specimens by using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse transcribed with Omniscript Reverse Transcriptase (Qiagen). RT-PCR was performed with the ABI PRISM 7700 (Applied Biosystems, Foster City, Calif). Measurement of the mRNA expression of hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) was performed in triplicate. The results are expressed after normalization for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Statistical Analysis

All data were expressed as the mean $\pm$ SEM and range. Student's unpaired *t* test or analysis of variance for parametric values was used to compare group means.

RESULTS

Pressure Overload and Hypertrophy of the Right Ventricle After PAB

After PAB, a weight analysis showed the heart weight/BW, RV/BW, and RV/(LV+IVS) weight ratios in the sham and

MST groups to be similar and significantly higher than in the control group (Table 1). Both the sham and MST groups showed a significantly increased end-systolic pressure and dP/dtmax than seen in the control group (Table 2).

Hemodynamic Effects of MST

The baseline indices revealed that end-diastolic pressure and τ values were significantly increased only in the sham group in comparison with those in the control group but not in the MST group (Table 2). Typical examples of the pressure–volume loop in each group are presented in Figure 1. The pressure–volume loop analysis revealed that the ESPVR and PRSW values significantly increased both in the sham and MST groups. However, the EDPVR value significantly increased only in the sham group (control vs sham vs MST groups: 8.6 ± 2.9 vs 16.1 ± 4.5 vs 7.6 ± 2.4 mL; $P < .05$ in the control and MST groups vs the sham group; Table 2).

Histologic Effects of MST

Whole heart findings showed the RV wall thickened, the cavity enlarged, and the IVS shifted toward the left side in the sham and MST groups (Figure 2, A). In the MST group transplanted MSs were observed as an elastic thin layer on the epicardium (Figure 2, B). The RV wall thickness and myocardial cell size in the sham and MST groups were similar and significantly higher than in the control group (Figure 2, C–E).

TABLE 1. Weight analysis at the fourth week after the operation

Group	Control	Sham	MST
HW/BW (mg/g)	2.62 ± 0.09	$3.53 \pm 0.50^*$	$4.03 \pm 0.59^*$
RV/BW (mg/g)	0.54 ± 0.15	$1.62 \pm 0.42^*$	$1.65 \pm 0.32^*$
RV/(IVS+LV)	0.27 ± 0.08	$0.69 \pm 0.11^*$	$0.69 \pm 0.09^*$

MST, Myoblast sheet transplantation; HW, heart weight; BW, body weight; RV, right ventricle; IVS, interventricular septum; LV, left ventricle. $P < .05$ versus the control group.

TABLE 2. Hemodynamic indices at the fourth week after the operation

Group	Control	Sham	MST
Basic hemodynamic indices			
HR (beats/min)	280 ± 72	233 ± 34	249 ± 65
ESP (mm Hg)	22.8 ± 2.9	82.3 ± 11.8*	78.7 ± 13.2*
EDP (mm Hg)	2.4 ± 1.4	10.3 ± 3.1*†	5.0 ± 3.7
dP/dtmax (mm Hg/s)	1301 ± 206	3197 ± 597*	3352 ± 1332*
dP/dtmin (mm Hg/s)	−997 ± 210	−2466 ± 582*	−2682 ± 828*
τ (ms)	7.9 ± 2.7	11.1 ± 2.5*†	7.6 ± 1.2
Load-independent parameters analyzed by PV loop			
ESPVR (mm Hg/mL)	538 ± 196	857 ± 305*	967 ± 201*
EDPVR (/mL)	8.6 ± 2.9	16.1 ± 4.5*†	7.6 ± 2.4
PRSW (mm Hg)	17.0 ± 4.1	40.2 ± 19.6*	40.8 ± 13.6*
PRSW/RV (mm Hg/kg)	88.3 ± 23.9	71.5 ± 31.3	73.6 ± 28.8

MST, Myoblast sheet transplantation; HR, heart rate; ESP, end-systolic pressure; EDP, end-diastolic pressure; PV, pressure–volume; ESPVR, end-systolic pressure–volume relationship; EDPVR, end-diastolic pressure–volume relationship; PRSW, preload-recruitable stroke work; RV, right ventricular weight. **P* < .05 versus the control group. †*P* < .05 versus the MST group.

Factor VIII stain showed that myocardial capillary vascular density showed no significant difference at the mid layer and endocardial layer (Figure 3, B and C), but it was significantly higher in the MST group than in the other 2 groups at the epicardial layer (Figure 3, A). Hence, total capillary density in the MST group was significantly higher than in the

other 2 groups (control vs sham vs MST groups: 262 ± 98 vs 271 ± 289 vs 823 ± 708; *P* < .05 in the control and sham groups vs the MST group; Figure 3, D).

Periodic acid–Schiff staining demonstrated significant interstitial fibrosis of the right ventricle in both the sham and MST groups, but the percentage of fibrosis in the

FIGURE 2. Macroscopic photographs of hematoxylin and eosin–stained sections showing right ventricular (RV) wall thickening, cavity enlarging, and the intraventricular septum shifting toward the left side in the sham and myoblast sheet transplantation (MST) groups (A and D). Photomicrographs (40×, scale bar = 200 μm) of hematoxylin and eosin–stained sections showed a fibrous organized thin layer on the epicardium in the myoblast sheet transplantation group (B). Photomicrographs (400×, scale bar = 20 μm) of periodic acid–Schiff–stained sections showed significantly hypertrophied ventricular myocytes in the sham and myoblast sheet transplantation groups (C and E). **P* < .05 (*n* = 10).

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FIGURE 3. Representative photomicrographs (100 $\times$, scale bar = 100 μ m) of Factor VIII–stained epicardial layer (A), mid layer (B), and endocardial layer (C). Neovascularization occurred at the epicardial layer in the myoblast sheet transplantation (MST) group (D). * $P < .05$ (n = 10).

MST group was significantly less than that in the sham group (control vs sham vs MST groups: $4.8\% \pm 1.1\%$ vs $24.5\% \pm 10.0\%$ vs $19.0\% \pm 5.1\%$; $P < .05$ between each 2 groups; Figure 4, A and E). Aggregated endomyocardial fibrosis was detected only in the sham group (endomyocardial percentage of fibrosis, control vs sham vs MST groups: $5.7\% \pm 0.1\%$ vs $31.5\% \pm 8.4\%$ vs $18.6\% \pm 5.9\%$; $P < .01$ in the control and MST groups vs the sham group; Figure 4, B–D and F).

RT-PCR

The expression of HGF and VEGF mRNA in the MST group was significantly higher than in the sham group (control vs sham vs MST groups: HGF, 0.00009 ± 0.00008 vs 0.00041 ± 0.00030 vs 0.00073 ± 0.00031 /GAPDH [$P < .05$ in each group]; VEGF, 0.00242 ± 0.00164 vs 0.00329 ± 0.00181 vs 0.00512 ± 0.00113 /GAPDH [$P < .05$ in the control and sham group vs the MST group]; Figure 5).

DISCUSSION

This study demonstrated that skeletal MST improved diastolic function in a pressure-overloaded right heart model in rats by means of PAB. This conclusion is supported by the following evidence: (1) the diastolic function was significantly improved based on hemodynamic assessment and pressure–volume loop analysis; (2) interstitial and endocardial fibrosis was ameliorated, and capillary vascular density of the epicardial layer was increased; and (3) myocardial

gene expression of HGF and VEGF was significantly increased. MST has been shown to have therapeutic effects in several models of LV failure.^{14–16} However, the present results are the first to show evidence that MST is effective for the treatment of RV dysfunction resulting from chronic pressure overload.

Prolonged RV pressure overload promotes unique morphologic, histologic, and functional changes. The mechanical stimulation of pressure overload extends the myocardium, which leads to diastolic dysfunction.²¹ Simultaneously, hypertrophied myocardium upregulates the release of various chemical mediators,¹⁷ which induce further myocardial expansion, apoptosis, necrosis, and fibrosis, finally resulting in RV decompensation. Otherwise, ventricular hypertrophy itself reduces the coronary flow reserve (CFR) and leads to coronary microcirculatory dysfunction.²⁵ As studies on the left ventricle show, this phenomenon is detected particularly in the subendocardium.²⁶ The shortage of CFR induces myocardial cellular mortality and endocardial fibrosis, which accelerates ventricular dysfunction. The present and previous data indicate that transplanted elastic cells initially improved ventricular stiffness¹⁴ and preserved CFR in the hypertrophied myocardium, both of which generate a synergistic effect of the suppression of myocardial cell death and fibrosis, especially at the endocardial layer. Although an angiogenic effect was observed with the increased myocardial gene expression of HGF and VEGF, we speculate this does not increase endocardial coronary flow directly because increased

FIGURE 4. Representative photomicrographs of Masson's trichrome–stained transmurals (A; 40×, scale bar = 200 μm), epicardial layer (B), mid layer (C), and endocardial layer (D; 200×, scale bar = 50 μm) for evaluation of interstitial (E) and endomyocardial (F) fibrosis. **P* < .05 (n = 10).

capillary density was only seen at epicardial layer. These new capillaries might increase the blood supply to the transplanted myoblasts and prolong their survival.¹³

As several LV studies have previously shown, Lamberts and colleagues²⁷ revealed the strict relationship between RV chamber stiffness and the degree of myocardial fibrosis. In their report they also described that prevention or reduction of RV fibrosis improved RV diastolic dysfunction. Although we could not show any individual correlation between collagen contents and hemodynamic parameters in regard to diastolic function, these findings strongly support our results. Therefore we would like to emphasize that the suppression of fibrosis improved the RV diastolic function.

The hemodynamic assessment of cardiac performance of a hypertrophied right ventricle has not been established. Various attempts have been made; however, it is still necessary to perform cardiac catheterization to evaluate hemodynamics, especially regarding diastolic function.^{28,29} Leeuwenburgh and associates⁴ showed that RV compliance deteriorated in a lamb model and described τ and EDPVR values to be useful indicators for diastolic function. Gaynor

FIGURE 5. RT-PCR for evaluation of neurohormonal factor gene expression. The results are expressed after normalization for GAPDH. *HGF*, Hepatocyte growth factor; *VEGF*, vascular endothelial growth factor. **P* < .05 (n = 10).

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and coworkers⁵ also showed an increase of EDPVR in a dog model of PAB. The current results are consistent with these reports. Therefore these data appear to be reliable.

On the other hand, the assessment of systolic function still remains controversial. In this study we showed that ESPVR (Ees) and PRSW were significantly increased in sham groups, as previously reported.³⁰ However, Dell'Italia and Walsh³¹ pointed out that in the assessment of RV contractility, the slope of ESPVR (Ees) was different from the slope of the maximum time-varying elastance, which better reflected the RV contractility than Ees. In addition to Ees, PRSW is thought to be an optimal parameter of ventricular contractility. However, we might have to consider the discrepancy of the cardiac mass between normal and hypertrophied hearts because ventricular SW should be assessed as a per-unit cardiac mass. We tried to calculate the PRSW divided for each animal's RV weight, which revealed there was no statistical significance among the 3 groups (control vs sham vs MST groups: 88.3 ± 23.9 vs 71.5 ± 31.3 vs 73.6 ± 28.8 mm Hg/kg, Table 2). Hence it is hard to say that the RV pressure load induced a further improvement in RV contractility.

We need further investigation to apply this method to clinical RV failure because we did not ascertain the effect of MST on RV systolic function. Nevertheless, enormous fibrosis was seen in the sham group, and systolic function was still compensated until the timing of hemodynamic evaluation. However, previous reports showed that MST had an excellent effect on LV contractility.¹⁴⁻¹⁶ Therefore we expect that this method might become a novel and potentially effective treatment strategy for patients with RV failure.

In conclusion, chronic pressure overload to the right ventricle caused hypertrophy and impaired diastolic function in rats. Skeletal MST attenuated diastolic dysfunction, which was mainly caused by suppressed interstitial and endocardial fibrosis. This method might become a novel strategy for the myocardial regeneration of RV failure in patients with CHD in the future.

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